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Research Paper

Detection of *Campylobacter jejuni* from Fresh Produce: Comparison of Culture- and PCR-based Techniques, and Metagenomic Approach for Analyses of the Microbiome before and after Enrichment

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ABSTRACT

In this study, we compared the efficiency of culture-based methods with or without membrane filtration, real-time PCR, and digital droplet PCR (ddPCR) for the detection of *Campylobacter* in fresh produce. Alfalfa sprouts, clover sprouts, coleslaw, and lettuce salad spiked with *Campylobacter jejuni* were enriched in Bolton broth for 48 h, and enrichment cultures were either directly inoculated onto modified charcoal-cefoperazone-deoxycholate agar or applied on membrane filters placed on the surface of plating media. In parallel, 2-mL Bolton broth cultures were taken to extract DNA for real-time PCR and ddPCR assays and bacterial community analysis. A developed primer set for ddPCR and real-time PCR was evaluated for its inclusivity and exclusivity using pure culture of *C. jejuni* and non–*C. jejuni* strains, respectively. In pure culture, the primer set reacted only with *C. jejuni* strains and showed negative reaction to non–*C. jejuni* strains. There was no significant difference ($P > 0.05$) in the detection efficiency of positive *Campylobacter* isolates from coleslaw and lettuce salad using four detection methods. However, for sprout samples, the detection efficiency of the culture method was significantly ($P < 0.05$) lower than those of the two PCR assays and the filtration method. The analysis also revealed the presence of *Pseudomonas* and *Acinetobacter* as the most prevalent competing microbiota in enriched culture and only *Acinetobacter* on agar plates in the selective culture step.

HIGHLIGHTS

- Culture method showed inferior detection ability to PCR and filtration in sprouts.
- Filter method showed similar detection ability to PCR in all samples.
- *Pseudomonas* and *Acinetobacter* are common competing flora in the enrichment step.

Key words: *Campylobacter jejuni*; Filtration; Metagenomics; PCR; Produce

Campylobacter spp. are microaerophilic bacteria that cause food poisoning worldwide, and *Campylobacter jejuni,* among all *Campylobacter* species, accounts for around 90% of campylobacteriosis cases *(7, 44)*. Although poultry meats are the primary sources of campylobacteriosis, outbreaks of *Campylobacter* infection have been associated with contaminated fruits, vegetables, or other produce-related products *(7, 13, 31, 33)*. The origin of contamination of these leafy vegetables is poultry manure used as fertilizer in vegetable gardens and irrigation water used, as well as cross-contamination in domestic or catering kitchens *(21, 34)*.

The rise in health consciousness among people has resulted in an increase in the consumption of fresh produce, such as sprouts and ready-to-eat (RTE) vegetables, which are potential sources of foodborne pathogens *(10). C. jejuni* is the third most common bacterium causing food poisoning through fresh produce consumption in the United States, after *Salmonella* spp. and *Escherichia coli* O157:H7 *(8)*. In a retrospective cohort study performed in the United Kingdom, Evans et al. *(23)* found that vegetables were the second most common risk factor, after chicken, in cases of *Campylobacter* infection. Even when the infective dose of the pathogen is low (approximately 500 CFU) *(6, 46),*

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consumption of contaminated uncooked fresh produce may cause campylobacteriosis *(11, 12, 33)*. Moreover, cross-contamination and temperature abuse during storage can enhance the risk represented by low levels of pathogen contamination *(18, 33)*. Because regular monitoring of pathogens in fresh produce is essential for consumer health and safety, the occurrence of *Campylobacter* in vegetables has been the subject of many studies *(8, 10, 12, 13, 22, 33, 35, 44, 48, 52, 53)*. However, isolation of *C. jejuni* from fresh produce using conventional culture methods is challenging compared with isolation from other food samples because of the low number of target bacteria in this matrix and the exponential growth of diverse indigenous microbiota during the enrichment step, which generates false positives on selective agar *(3, 13, 25, 42)*. Indigenous flora grown via selective enrichment steps can mask target pathogens, making differentiation and isolation of suspicious colonies difficult. Considering that the causative pathogens in more than half of cases of food poisoning outbreaks in the United States remain unknown *(9, 55),* the potential risk and prevalence of *Campylobacter* in fresh produce may be underestimated because of the low reliability of the current detection methods *(13, 59)*. Even though the number of reported cases of campylobacteriosis and prevalence of the bacteria in fresh produce have been lower compared with those in poultry meat, we cannot be sure whether the potential risk and actual prevalence are negligible, because the current detection method is not reliable in detecting *Campylobacter* in fresh produce.

The level of indigenous microbiota is high and diverse in fresh produce *(28)*. In particular, the indigenous microbiota of ready-to-eat sprouts is extremely complex and diverse, because the optimal growth condition for them is ideal for the growth of bacteria *(13, 20, 28, 40)*. Even though *Campylobacter* enrichment broth has various antibiotics as a supplement, it has failed to exclude competing flora properly in the enrichment step in fresh produce *(13)*. It allows the overgrowth of competing flora, masking the growth of the target bacteria. However, the composition of the competing flora during *Campylobacter* enrichment in fresh produce has not been studied in previous research. Information on the dominant bacteria in the enrichment step can be useful in improving the selective media that are used in the official culture method.

The direct application of membrane filters to the surface of the plating media has been previously used to eliminate unwanted microbiota and enable selective isolation of *Campylobacter (50, 51)*. Because *Campylobacter* is characterized by low width (0.2 to 0.8 μm) and length (0.5 to 5.0 μm) and corkscrew-like motility with spiral morphology, the pathogen can traverse 0.45- to 0.65-μm-pore-size filters and this method has successfully been used to recover *Campylobacter* spp. from poultry, clinical, and water samples *(2, 15, 19, 50, 58)*. However, this method has not yet been implemented for isolating the pathogen from the complex biological matrix of fresh produce that also harbors other indigenous microbiota.

Rapid molecular biology–based detection methods have been applied for the screening and isolation of foodborne pathogens *(36, 37, 57)*. PCR is considered superior to conventional culture methods for detecting pathogens in fresh produce, because the presence of indigenous microbiota has a negligible effect on PCR performance *(28, 39)*. Real-time PCR detects amplified DNA as a fluorescent signal and does not require gel electrophoresis for visualization of the amplified fragments, which renders the method convenient and highly sensitive *(36)*. Recently, a new method called digital droplet PCR (ddPCR) was developed, in which DNA samples are digested with restriction enzyme and partitioned into water-in-oil droplets (approximately 20,000 droplets), each of which acts as an individual reaction *(30, 41)*. The amplification is monitored by a platform, which determines whether each droplet is either positive or negative for the presence of a pathogen based on the fluorescent signal *(24, 30)*. The ddPCR has been used to detect *C. jejuni* in water and cheese but not in fresh produce *(17, 47)*.

In the present study, we compared the conventional culture method, membrane filter method, real-time PCR, and ddPCR for the selective detection of *C. jejuni* from four types of fresh produce samples. In addition, we analyzed the bacteria before and after the enrichment step to determine changes in microbial community composition during the detection process.

MATERIALS AND METHODS

Bacterial strains. The 25 bacterial strains (6 *C. jejuni* and 19 non–*C. jejuni*) used in this study are presented in Table 1. The cultures were streaked onto brain heart infusion (BHI) agar (Difco, BD, Sparks, MD) with 5% laked horse blood (BHI-blood agar; Oxoid, Hampshire, UK), followed by incubation at 37°C for 48 h in either an aerobic (for non-*Campylobacter* genus) or a microaerobic (for *Campylobacter* genus) atmosphere containing 5% O_2, 10% CO_2, and 85% N_2. Bacterial genomic DNA templates were extracted from colonies with a boiling method. Single colonies were removed from the culture plate and mixed with 1 mL of phosphate-buffered saline (PBS; Sigma-Aldrich, St. Louis, MO), followed by centrifugation at 20,187 × *g* for 3 min. The pellets were resuspended in 500 μL of PBS and boiled for 10 min. The samples were centrifuged at 20,187 × *g* for 3 min. The supernatant was used for real-time PCR and ddPCR of pure cultures.

Artificial inoculation of *C. jejuni* in produce samples. Four types of vegetables—alfalfa sprouts, clover sprouts, RTE coleslaw (made of sliced green cabbage), and RTE iceberg lettuce salad (composed of iceberg lettuce, red cabbage, and carrots)—were purchased from a retail store in Little Rock, AR. *C. jejuni* NCTC 11168 was used for artificial inoculation in the present study. The stock culture was inoculated onto BHI-blood agar and incubated at 42°C for 48 h under microaerobic conditions. A single colony was removed from the plate and inoculated into Bolton broth (Oxoid), followed by incubation at 42°C for 24 h under microaerobic conditions. Bolton broth was serially diluted in PBS, and each dilution was inoculated onto BHI-blood agar. Plates were incubated at 42°C for 24 h to enumerate the number of cells in Bolton broth. Based on the culturable *C. jejuni* counts, Bolton broth was diluted and the appropriate CFU (less than 10^1, 10^1 to 10^2, 10^2 to 10^3, and 10^3 to 10^4 CFU/50 g of produce) of *C. jejuni* NCTC 11168 was spiked into vegetable samples. Five replicates were used per concentration. The number of CFU inoculated into each vegetable sample was confirmed by plate counting on BHI-blood agar. In addition, an uninoculated (50 g) and a highly

TABLE 1. *Inclusivity and exclusivity of real-time PCR and ddPCR data for the detection of pure cultures of* C. jejuni *and non–*C. jejuni *strains*

Species	Strain	Reaction	
		Real-time PCR	ddPCR
Campylobacter jejuni	*C. jejuni* NCTC 11168	+	+
	C. jejuni 81-176	+	+
	C. jejuni A74C	+	+
	C. jejuni 4549	+	+
	C. jejuni SC_Cj5	+	+
	C. jejuni SC_Cj7	+	+
Non–*C. jejuni*	*C. coli* ATCC 33559	−	−
	C. sputorum ATCC 33562	−	−
	C. showae ATCC 51146	−	−
	E. coli ATCC 25922	−	−
	Salmonella Enteritidis 22079	−	−
	Shigella sonnei ATCC 9290	−	−
	Yersinia enterocolitica ATCC 27729	−	−
	Enterobacter aerogenes ATCC 13048	−	−
	Citrobacter freundii ATCC 8090	−	−
	Bacillus cereus ATCC 21772	−	−
	Bacillus subtilis ATCC 6051	−	−
	Staphylococcus aureus ATCC 25923	−	−
	Staphylococcus epidermidis ATCC 12228	−	−
	Lactobacillus salivarius ATCC 11741	−	−
	Pseudomonas aeruginosa ATCC 7853	−	−
	Enterococcus faecalis ATCC 29212	−	−
	Proteus mirabilis ATCC 7002	−	−
	Acinetobacter spp. ACB3	−	−
	Ochrobactrum anthropi HAC7	−	−

inoculated (more than 10^7 CFU/50 g) food sample were used as a negative and a positive control, respectively. A mesophilic aerobic plate count was performed on the uninoculated food sample, which was homogenized with 100 mL of Butterfield's phosphate-buffered water (Difco). Each homogenate (1 mL) was serially diluted with PBS, and each dilution was inoculated on duplicated Petrifilm Aerobic Count plates (3M, Minneapolis, MN). The inoculated films were incubated at 37°C for 48 h, followed by enumeration of aerobic mesophilic bacteria. Two milliliters of sample with no inoculation (homogenates in Bolton broth) was collected into microcentrifuge tubes, and DNA extracted from the samples was used for the analysis of the fresh produce–derived bacterial community.

Real-time PCR. In a preliminary study, several primer set candidates from various *C. jejuni*–specific genes were evaluated with the small number of *C. jejuni* and non–*C. jejuni* strains to find the best primer sequences, and a candidate that showed the best performance (C_T [cycle threshold] value, inclusivity-exclusivity, etc.) was selected. The sequences of the genes were extracted from GenBank (http://www.ncbi.nlm.nih.gov/Genbank/), and several primer sets were designed using the PrimerQuest tool of Integrated DNA Technologies (Coralville, IA). The sets were validated at the National Center for Biotechnology Information (NCBI) Web site (http://www.ncbi.nlm.nih.gov/blast/) using Basic Local Alignment Search Tool standard databases (nucleotide collection). The primer sequence targeting *hipO* gene (accession no. FJ655193.1) was used for further study. The sequences were as follows: amplicon size of 105 bp; forward primer, 5′-AGCAAAGAAGCAGCA-TAAATAGG-3′; reverse primer, 5′-GATGATGGCTTCTTCGGA-TAGT-3′. The extracted DNA (5 μL) was transferred into a PCR tube containing 10 μL of SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA), 2 μL of forward primer (500 nM) and 2 μL of reverse primer (500 nM), and 1 μL of nuclease- and protease-free water. The eight-multiwell tubes (Bio-Rad Laboratories) were placed in a CFX96 real-time PCR machine (Bio-Rad Laboratories). The reaction was run at 98°C for 2 min, 40 cycles of 98°C for 5 s, 60°C for 5 s, followed by 65 to 95°C running for 10 s in each step in increments of 0.5°C for the generation of melting curve.

ddPCR. The *hipO* gene was detected using a QX100 Droplet Digital PCR system (Bio-Rad Laboratories). The reaction mixture was composed of 5 μL of DNA template, 10 μL of 2× QX200 ddPCR EvaGreen Supermix (Bio-Rad Laboratories), 2 μL of forward primer (250 nM), 2 μL of reverse primer (100 nM), and 1 μL *Hae*III restriction enzyme. To generate the droplets, 20 μL of ddPCR mixture and 70 μL of droplet generation oil (Bio-Rad Laboratories) were inserted in an eight-well cartridge covered with a rubber gasket, followed by placement in the QX200 droplet generator (Bio-Rad Laboratories). A total of 40 μL of the generated droplets were transferred into a 96-well PCR plate (Eppendorf, Hamburg, Germany) followed by amplification in a T100 thermal cycler (Bio-Rad Laboratories). Cycling conditions for amplification started with 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 1 min at 60°C, and then 10 min at 98°C for droplet stabilization and 4°C for cooling. After the PCR reaction, 96-well PCR plates were transferred to a droplet reader (Bio-Rad Laboratories) to detect the fluorescent intensities in droplets. QuantaSoft 1.3.2.0 software (Bio-Rad Laboratories) was used to analyze the concentration of DNA from positive reactions using Poisson distribution *(41)*.

TABLE 2. *Comparison of positive results for* C. jejuni *using the four detection methods in four types of fresh produce*

Sample	Inoculum (CFU/50 g)	No. of positives/total no. tested (%)[a]			
		Culture method[b]	Filtration method[c]	Real-time PCR	ddPCR
Alfalfa sprouts	Less than 10^1	0/5	0/5	0/5	0/5
	10^1–10^2	0/5	2/5	3/5	3/5
	10^2–10^3	1/5	4/5	5/5	5/5
	10^3–10^4	0/5	4/5	4/5	4/5
Subtotal		1/20 A	10/20 B	12/20 B	12/20 B
Clover sprouts	Less than 10^1	0/5	3/5	4/5	4/5
	10^1–10^2	1/5	5/5	5/5	5/5
	10^2–10^3	0/5	5/5	5/5	5/5
	10^3–10^4	3/5	5/5	5/5	5/5
Subtotal		4/20 A	18/20 B	19/20 B	19/20 B
Coleslaw	Less than 10^1	3/5	3/5	4/5	4/5
	10^1–10^2	4/5	5/5	5/5	5/5
	10^2–10^3	5/5	5/5	5/5	5/5
	10^3–10^4	3/5	5/5	5/5	5/5
Subtotal		15/20 A	18/20 A	19/20 A	19/20 A
Lettuce salad	Less than 10^1	4/5	3/5	4/5	4/5
	10^1–10^2	5/5	4/5	5/5	5/5
	10^2–10^3	5/5	4/5	5/5	5/5
	10^3–10^4	5/5	5/5	5/5	5/5
Subtotal		19/20 A	16/20 A	19/20 A	19/20 A
Total[a]		39/80 A	62/80 B	69/80 B	69/80 B

[a] The number of positive plates for *C. jejuni* was statistically evaluated by Fisher's exact test using GraphPad Instat software (GraphPad Software, San Diego, CA). Different letters (A and B) within a row indicate a significant difference ($P < 0.05$) in the number of positives.
[b] FDA BAM.
[c] Modified FDA BAM.

Inclusivity-exclusivity test and detection limit of PCR for pure cultures. To determine the inclusivity and/or exclusivity of the designed sequences, real-time PCR and ddPCR were used to examine 6 *C. jejuni* and 19 non–*C. jejuni* strains. Non–*C. jejuni* strains included non–*C. jejuni Campylobacter* species and common pathogens that can be found in food, such as *Salmonella, Yersinia enterocolitica, Bacillus cereus,* and *Staphylococcus aureus*. *Acinetobacter, Ochrobactrum, Pseudomonas, Lactobacillus,* and *E. coli* were also included in the test, because those are common bacteria that can concomitantly grow in *Campylobacter* selective broth during the enrichment process *(1, 3, 14, 26, 42, 54, 59)*. To determine the detection limits of real-time PCR and ddPCR, genomic DNA was extracted according to the method described in the "Bacterial strains" section. The extracted DNA was serially diluted (10-fold) in PBS, and real-time PCR and ddPCR were performed with each dilution. The lowest number of bacteria showing positive results was determined as the detection limit of the PCR assays.

Barcoded pyrosequencing for bacterial community analysis for samples before and after the enrichment. The bacterial community analysis of collected culture was conducted using barcoded pyrosequencing according to a previous study *(32)*. The extracted DNA from unenriched and enriched samples stated in "Artificial inoculation of *C. jejuni* in produce samples" were used for pyrosequencing; each unenriched sample and six enriched samples of four vegetable types were selected, considering the inoculation level (alfalfa sprouts no. 4, 5, 7, 8, 13, and 20; clover sprouts no. 4, 5, 8, 9, 15, and 20; coleslaw no. 2, 3, 7, 8, 12 and 18; lettuce salad no. 3, 7, 9, 15, 16, and 20; Table 2). Six samples from each vegetable type were randomly selected for the sequencing. The six samples should include each inoculation level (less than 10^1, 10^1 to 10^2, 10^2 to 10^3, and 10^3 to 10^4 CFU/50 g). A primer set, BacF (5′-adaptor B-AC-9F-3′)/BacR (5′-adaptor A-X-AC-541R-3′) *(37),* where X denotes unique 7 to 11 barcoded sequences inserted between the 454 Life Sciences adaptor A sequence and a common linker AG, was used for the amplification of bacterial 16S rRNA (V1 to V3 variable regions). All PCR amplifications were performed in a MyCycler (Bio-Rad Laboratories) with a 50-μL volume containing a DreamTaq DNA polymerase mixture (K1072; Thermo Fisher Scientific, Wilmington, DE), 1 μL of genomic DNA, and 20 pmol of each primer. The cycling regime was as follows: 94°C for 5 min, 1 cycle; 94°C for 45 s, 55°C for 45 s, and 72°C for 1 min, 30 cycles; and 72°C for 10 min, 1 cycle. The PCR amplicons were purified using a PCR purification kit (Qiagen, Hilden, Germany), and the concentrations were carefully measured with a NanoDrop 1000 (Thermo Fisher Scientific). An equal amount (300 ng) of each purified PCR product was pooled for pyrosequencing, followed by sequencing with a 454 GS-FLX titanium platform (Roche, Penzberg, Germany) at Macrogen Corp. (Rockville, MD). Bacterial sequencing reads generated by pyrosequencing were processed and analyzed using the RDPipeline (http://pyro.cme.msu.edu/) *(16)*. The sequencing reads were sorted into specific samples based on their unique barcoded sequences, and the barcodes were trimmed using the Pipeline Initial Process in RDPipeline. Sequencing reads with more than two ambiguous base calls (*n*), shorter than 300 bp, or with average quality scores under 25 (error, 0.005) were removed from the process. Putative chimeric sequencing reads were excluded by the de novo chimera detection function UCHIME in USEARCH of RDPipeline. The processed bacterial sequencing reads were divided into hierarchical bacterial taxa at the phylum and genus levels with an 80% confidence cutoff using the Ribosomal Database Project (RDP) naïve Bayesian

TABLE 3. *The number of genera and dominant competing microbiota during* C. jejuni *isolation from fresh produce*[a]

Sample (no. of indigenous microbiota, log CFU/g)	Sample no.	Inoculum (CFU/50 g)	Primary and secondary dominant competing bacteria in unenriched and enriched samples (relative abundance, %)	Primary dominant competing bacteria on mCCDA
Alfalfa sprouts (7.62)	Unenriched	NA	*Flavobacterium* (20), *Pseudomonas* (18)	NA
	4	Less than 10^1	*Pseudomonas* (67), *Acinetobacter* (15)	ND
	5	Less than 10^1	*Pseudomonas* (60), *Acinetobacter* (13)	ND
	7	10^1–10^2	*Pseudomonas* (78), unclassified *Pseudomonadaceae* (4)	ND
	8	10^1–10^2	*Pseudomonas* (61), *Acinetobacter* (13)	*Acinetobacter*
	13	10^1–10^2	*Pseudomonas* (60), *Acinetobacter* (15)	*Acinetobacter*
	20	10^3–10^4	*Pseudomonas* (89), unclassified *Pseudomonadaceae* (4)	*Acinetobacter*
Clover sprouts (7.63)	Unenriched	NA	*Yersinia* (46), *Streptophyta* (23)	NA
	4	Less than 10^1	*Pseudomonas* (35), *Acrobacter* (31)	ND
	5	Less than 10^1	*Pseudomonas* (42), unclassified *Enterobacteriaceae* (13)	ND
	8	10^1–10^2	*Pseudomonas* (32), *Acrobacter* (24)	*Acinetobacter*
	9	10^1–10^2	*Pseudomonas* (69), unclassified *Enterobacteriaceae* (10)	ND
	15	10^1–10^2	*Acinetobacter* (56), unclassified *Enterobacteriaceae* (16)	*Acinetobacter*
	20	10^3–10^4	*Acinetobacter* (52), unclassified *Enterobacteriaceae* (15)	*Acinetobacter*
Coleslaw (8.20)	Unenriched	NA	*Pseudomonas* (48), *Pantoea* (12)	NA
	2	Less than 10^1	*Acinetobacter* (65), *Pseudomonas* (<1)	*Acinetobacter*
	3	Less than 10^1	*Acinetobacter* (99), *Pseudomonas* (<1)	ND
	7	10^1–10^2	*Acinetobacter* (73), *Pseudomonas* (<1)	*Acinetobacter*
	8	10^1–10^2	*Acinetobacter* (54), *Pseudomonas* (<1)	ND
	12	10^1–10^2	*Acinetobacter* (22), *Pseudomonas* (<1)	ND
	18	10^3–10^4	*Acinetobacter* (41), *Pseudomonas* (<1)	*Acinetobacter*
Lettuce salad (5.04)	Unenriched	NA	*Pseudomonas* (56), *Janthinobacterium* (7)	NA
	3	Less than 10^1	*Acrobacter* (72), *Pseudomonas* (22)	*Acinetobacter*
	7	10^1–10^2	*Pseudomonas* (99), unclassified *Pseudomonadaceae* (<1)	NC
	9	10^1–10^2	*Pseudomonas* (99), unclassified *Pseudomonadaceae* (<1)	NC
	15	10^1–10^2	*Pseudomonas* (62), unclassified *Pseudomonadaceae* (3)	NC
	16	10^3–10^4	*Pseudomonas* (47), unclassified *Pseudomonadaceae* (3)	NC
	20	10^3–10^4	*Pseudomonas* (98), unclassified *Pseudomonadaceae* (1)	NC

[a] NA, not applicable; ND, not determined; NC, no competing bacteria on plate.

classifier *(57)*. The pyrosequencing data of the 16S rRNA genes are publicly available in the NCBI Short Read Archive under accession no. SRX10063925 and SRX10511877-10511903.

Isolation of *C. jejuni* from spiked produce samples. The isolation of *C. jejuni* was conducted according to the method described in the *Bacteriological Analytical Manual* of the U.S. Food and Drug Administration (FDA BAM) with minor modification *(27)*. Each 50-g sample was put in a sterilized stomacher bag with 100 mL of Bolton broth (Oxoid) and homogenized for 30 s. After stomaching, the samples were preenriched in Bolton broth at 37°C for 4 h, followed by enrichment at 42°C for 44 h. A loopful of enrichment culture was streaked onto modified charcoal-cefoperazone-deoxycholate agar (mCCDA; Oxoid). For membrane filtration, 5 drops (20 μL each) of enrichment culture were spotted on a 0.65-μm-pore-size membrane filter (nitrocellulose membrane; Millipore, Schwalbach, Germany) on mCCDA agar, as previously described *(50)*. The membrane filter on the surface of a mCCDA plate was left for 15 to 20 min. All plates were incubated at 42°C for 48 h microaerobically. At least one suspected colony was removed from each of the plates and subcultured on BHI-blood agar, followed by incubation at 42°C for 48 h microaerobically. Putative isolates were confirmed using real-time PCR targeting *hipO* with DNA from suspected colonies. Two milliliters of enrichment broth was collected into microcentrifuge tubes and used for two PCR assays and microbiota sequencing of the enriched broth. Extraction of DNA templates was performed using the PowerSoil DNA isolation kit (MO BIO Laboratories, Solana Beach, CA), according to the manufacturer's instructions.

RESULTS

Inclusivity-exclusivity and detection limit of PCR assays for pure cultures. The inclusivity and exclusivity data of the used primers and probes for real-time PCR and ddPCR are shown in Table 1. Positive reactions were obtained with only six *C. jejuni* strains, not with any unrelated species. Both real-time PCR and ddPCR detected the DNA extracted from 10^1 colonies of *C. jejuni* NCTC 11168 for the matrix-free pure cultures. It indicates the developed primer set can selectively detect *C. jejuni* only, even with the presence of non–*C. jejuni* strains. This can be useful for *C. jejuni*–contaminated produce that has various background microbiota.

Comparison of the four methods for the isolation of *C. jejuni* from fresh produce. *C. jejuni* was not detected in the negative controls (uninoculated samples) using any tested isolation methods, whereas it was detected in all positive controls. This indicated that the samples were not naturally contaminated by *C. jejuni,* which excluded possible false positives. A comparison of the isolation rates of the four methods used in this study is presented in Table 3.

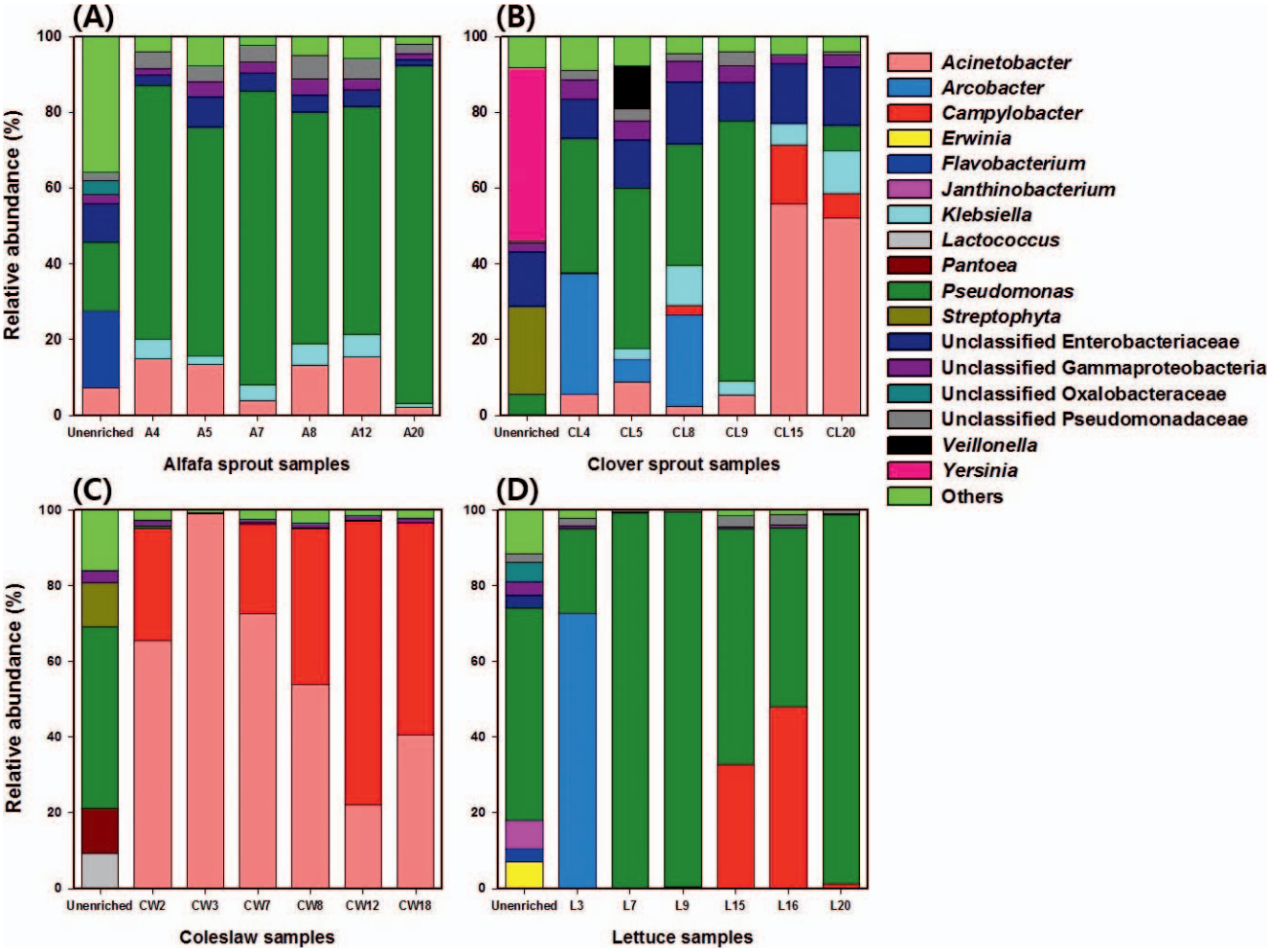

FIGURE 1. *Composition of the microbiomes in unenriched and enriched fresh produce samples. (A) Alfalfa sprouts, (B) clover sprouts, (C) coleslaw, (D) lettuce salad. 16S rRNA sequences were divided at the genus level using the MG-RAST server based on the RDP II (16S rRNA) database (*E *value, 0.01; minimum alignment length, 50 bp). Genera accounting for less than 5% of the community are marked as "other (black)."*

In tested produce samples, culture method (FDA BAM), filter method (modified FDA BAM), real-time PCR, and ddPCR showed the following number of positives: 39, 62, 69, and 69 of 80, respectively. Overall, the detection ability of the filtration method was significantly better ($P < 0.05$) than that of the culture method (39 versus 62 of 80), but slightly inferior ($P > 0.05$) to those of the two PCR assays (62 versus 69 of 80). The culture method exhibited a significantly lower ($P < 0.05$) number of positives compared with other tested methods. Although PCR assays showed slightly higher or similar numbers of positives compared with the culture-dependent methods, there was no significant difference ($P > 0.05$) in the number of positives in coleslaw or lettuce salad (Table 2). However, in sprouts samples, the detection ability of the culture method was significantly ($P < 0.05$) lower than those of real-time PCR, ddPCR, and the filtration method (Table 2). Both PCR methods provided the best detection ability, showing the same number of positives (69 of 80, 12 of 20, 19 of 20, 19 of 20, and 19 of 20 for alfalfa sprouts, clover sprouts, coleslaw, and lettuce salad, respectively) in all types of samples (Table 2).

Microbiota sequencing for detection of competing bacteria from the enriched broth. 16S rRNA gene-based barcoded pyrosequencing was applied to analyze the bacterial community of the enriched broth, and 166,148 sequencing reads were generated from the 28 samples. After removing low-quality reads including chimeric sequences, 144,760 reads (approximately 87.13% of total sequencing reads) with an average read length of approximately 469 bp and an average of 5,170 reads for each sample were finally used for bacterial community analysis (data not shown). The cell numbers of aerobic mesophilic bacteria present in each type of fresh produce and the most and second most prevalent competing bacterial genera found in the media are summarized in Table 3. Analyses of the microbiome before and after enrichment are also presented as bar graphs in Figure 1. Among the detected genus, *Pseudomonas* was the most common genus in homogenates in Bolton broth (unenriched) of alfalfa sprouts, coleslaw, and lettuce, whereas *Yersinia* was predominant in unenriched clover sprouts. In enriched medium, *Pseudomonas* and *Acinetobacter* were predominant in all samples. The number of indigenous bacteria determined by aerobic plate counts was

7.62, 7.63, 8.20, and 5.04 log CFU/g in alfalfa sprouts, clover sprouts, coleslaw, and lettuce salad, respectively. In enriched medium, *Pseudomonas* was still the most commonly observed bacterial genus in alfalfa sprouts (6 of 6), clover sprouts (4 of 6), and lettuce salad (5 of 6). *Acinetobacter,* which rarely appeared in unenriched samples, was predominantly observed as the primary contaminant in 2 of 6 of clover sprout samples and 6 of 6 of coleslaw samples or the secondary contaminant in 4 of 6 of alfalfa sprout samples in enriched Bolton broth (Table 3).

DISCUSSION

Detection of *Campylobacter jejuni* in complex food matrices is complicated by the slow growth of *C. jejuni* and the presence of exponentially growing non–*C. jejuni* contaminants during the enrichment step of the conventional culture method *(29, 42, 59)*. It seems that contamination with non–*C. jejuni* isolates was eliminated and *Campylobacter* isolation rate was restored when membrane filtration was applied before plating, especially in sprout samples. Reports show that the isolation of *Campylobacter* species from chicken samples using membrane filtration was greater than that without filtration, which is consistent with our results *(15, 58)*. Apart from membrane filtration, there are various other methods of excluding contaminating microbiota. For example, immunomagnetic separation can specifically capture the microorganism of interest on antibody-coated magnetic beads, which can be subsequently used for downstream experiments *(38, 56)*. Although specific, this method requires target-specific antibodies, which may limit its widespread use compared with the use of simple membrane filters.

Both PCR assays used here for the detection of *C. jejuni* from fresh produce were better than the culture-based methods in detecting positive isolates (Table 2). Previous studies reported that PCR was significantly superior to culture methods for the detection of foodborne pathogens, such as *Salmonella* and *S. aureus,* especially from fresh produce harboring diverse and complex indigenous microbiota *(28, 39),* which is consistent with our finding. Although both real-time PCR and ddPCR reliably detected *C. jejuni* from vegetables in this study, these methods are not as confirmative as culture-dependent methods *(31),* because the PCR assay can be inhibited by the presence of inhibitory compounds in food or by certain media components and are unable to distinguish between live and dead cells *(49)*. Therefore, the screening of *C. jejuni* in broth media using real-time PCR or ddPCR has to be followed by culture-based methods to conclusively determine the presence of the viable pathogen.

The enrichment step highly alters the taxonomic profiles of environmental and food samples, as well as that of the target organism *(4, 45)*. Certain indigenous bacteria in fresh produce may grow faster during the enrichment step and outcompete or even eliminate the target bacteria *(5, 43)*. The reason sprouts are more susceptible to contamination with non–*C. jejuni* isolates than coleslaw and lettuce salad in culture method could be related to the differences in the composition of the competing microbiota, rather than differences in the complexity of the food matrix or technical aspects of culturing (Table 3). This emphasizes the importance of evaluating microbiota shifts during the enrichment step. Reports on the existence of competing microbiota during the enrichment step are lacking, although certain studies confirmed the growth of contaminants in *Campylobacter*-selective agars, such as *Acinetobacter, Ochrobactrum, Pseudomonas, Lactobacillus,* and extended-spectrum β-lactamases–producing *E. coli,* while detecting *Campylobacter* from clinical, environmental, and food samples *(1, 3, 14, 26, 42, 54, 59)*. In this study, *Pseudomonas* and *Acinetobacter* were the most abundant competing bacterial genera in the enriched samples. We identified some competing microbiota that grew dominantly on mCCDA plates using 16S rRNA gene capillary sequencing. A total of 10 competing bacteria on mCCDA plates were collected, and all tested colonies were identified as *Acinetobacter,* regardless of the composition of the competing microbiota in the enrichment culture (Table 3). *Acinetobacter* predominantly appeared on agar plates even in samples containing *Pseudomonas* as the primary competing microbiota, such as in alfalfa sprouts no. 8, 13, and 20 (Table 3). *Acinetobacter* was not found in the amplicon sequencing data of the broth for lettuce sample no. 3 but was observed as the predominant competing genus on the agar plate. This change in taxonomic profile upon change of culture medium (from broth to agar) may be associated with the differences in the composition of selective and nonselective agents between Bolton broth and mCCDA, as well as variations within the indigenous bacterial community of the tested sample.

Possibly, the exponentially growing competing microbiota, which is relatively abundant compared with *C. jejuni* in Bolton broth containing various antibiotics, such as trimethoprim, cycloheximide, cefoperazone, and vancomycin, masks the presence of the target bacteria, especially in sprout samples (Fig. 1). Reports show that enrichment may not increase the probability of detecting a target bacterium despite the specific genetic or genomic method being employed because of the change in the bacterial communities of background microbiota, as well as the target organism *(4, 45)*. More rapid and efficient isolation methods should be developed and evaluated to increase recovery and selectivity for *Campylobacter* isolation from fresh produce. The screening of *C. jejuni* in broth media using real-time PCR or ddPCR, followed by confirmative detection and identification using the membrane filtration-culture method, would be optimum for confirming the presence of the viable pathogen in fresh produce, especially in sprout samples.

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