

## Efficacy of supplemented buffered peptone water for the isolation of *Campylobacter jejuni* and *C. coli* from broiler retail products

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### Abstract

Broiler retail samples ( $n=113$ ) were analyzed to determine (i) the effectiveness of buffered peptone water (BPW) supplemented with blood and antibiotics for the isolation of *Campylobacter jejuni* and *C. coli*, (ii) if a 1:4 enrichment ratio performs similarly as a 1:9 ratio, and (iii) if BPW is similar to Bolton broth for enumeration of *Campylobacter* spp. in retail broiler meat using the most probable number (MPN) procedure. Chi-square comparison showed that BPW performed similarly as Bolton broth ( $P \leq 0.05$ ) for *Campylobacter* isolation in breast tenders, boneless breasts, split breasts and skin samples. However, BPW showed a lower detection rate ( $P \geq 0.05$ ) for thighs and boneless thighs. When the results were combined, BPW performed similarly as Bolton broth for the isolation of *Campylobacter* spp. ( $P \leq 0.05$ ). BPW at an enrichment ratio of 1:4 was statistically similar to Bolton broth or BPW at a ratio of 1:9. No differences were observed between the MPN data from Bolton broth and the MPN data from BPW ( $P \leq 0.50$ ). A multiplex PCR assay revealed that ca. 48% of the isolates obtained from Bolton broth and 59% of the isolates obtained with BPW were *C. coli*. Both Bolton broth and BPW allowed for the growth of *C. jejuni* and *C. coli* from the same sample. Remarkably, a large genomic variability was observed by PFGE analysis of the isolates collected from the same sample with Bolton broth or BPW, which confirms that more than one genotype can successfully multiply during enrichment and be recoverable on agar plates. These findings suggest that BPW could be used as an enrichment medium for isolation of *Campylobacter* from retail broiler samples. The implications of the high number of *C. coli* isolates found in this study is discussed.

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### 1. Introduction

In the US, retail poultry meat samples are highly contaminated with *Campylobacter* spp., reported to be 69 (Willis and Murray, 1997), 71 (Zhao et al., 2001) and 82% (Dickins et al., 2002). As an alternative to enrichment, filtration and centrifugation techniques are commonly used to concentrate *Campylobacter* spp. cells in samples where the numbers of bacteria are low (<1 log), such as retail broiler meat samples,

and where direct quantification on agar plates is limited (Dickins et al., 2002; Willis and Murray, 1997). These limitations have been recently highlighted for the new quantitative method for *Campylobacter* by the International Organization for Standardization by the European Union (Hutchison et al., 2006).

Presently, a useful enrichment procedure of a retail broiler sample is needed to determine if the sample was contaminated with *Campylobacter* cells or not (Baylis et al., 2000; Kramer et al., 2000; Willis and Murray, 1997). Several enrichment broths have been developed for the isolation of *Campylobacter* from retail poultry meat (Corry et al., 1995; Doyle and Roman, 1981; Hunt et al., 2001). In general, enrichment broths contain some of the components used originally in the development of

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plate media for isolation of *Campylobacter* spp. from fecal samples in humans. The typical composition of an enrichment broth comprises a rich, basal medium, such as Brucella broth or Nutrient broth (Bolton and Robertson, 1982; Corry et al., 1995), antimicrobials, and the addition of lysed horse or sheep blood. The basal medium is added under the assumption that thermotolerant *Campylobacter* spp. are “fastidious” pathogens that require complex media to grow (Corry et al., 1995). However, we have an incomplete understanding of how other basal media, such as buffered peptone water (BPW), could perform for the isolation of *Campylobacter* spp. from retail meat. Bolton and Robertson (1982) used nutrient broth No. 2, peptone and sodium chloride, to develop Preston enrichment broth. However, poultry meat samples enriched in Preston broth exhibited less *Campylobacter* growth than Bolton broth and *Campylobacter* enrichment broth (Baylis et al., 2000). A blood-free enrichment medium with a composition similar to that of Bolton broth has been tested for the growth and isolation of *Campylobacter* spp. under aerobic condition and different food samples. The efficacy of this medium was affected by the food type and bacterial strain (Tran, 1998), which highlighted our lack of understanding of the impact of each component of a medium on the efficacy to isolate *Campylobacter* from food samples.

Buffered peptone water (BPW) is the medium recommended by the U. S. Department of Agriculture Food Safety and Inspection Services for the collection of microbiology samples with the rinse method from broiler carcasses (Anonymous, 1996). A successful enrichment with BPW could help with a direct enrichment of carcass rinses collected from processed broilers for the isolation of *Campylobacter* spp. Yet, such a medium must be validated with samples containing relatively low numbers of *Campylobacter* cells. The objectives of this study were: (i) to study the effectiveness of BPW supplemented with blood and antibiotics for the isolation of thermotolerant *Campylobacter* spp. from retail broiler meat; (ii) to determine if a 1:4 enrichment ratio performs similarly as a 1:9 ratio; and (iii) to assess if BPW is similar to Bolton broth for enumeration of thermotolerant *Campylobacter* spp. in retail broiler meat using the most probably number procedure. Isolates were identified with a multiplex polymerase chain reaction (PCR) assay and the extent of the DNA variability of isolates from the same sample was determined using pulsed field gel electrophoresis (PFGE).

## 2. Materials and methods

### 2.1. Sample collection

A total of 113 broiler retail samples were purchased from different retail stores in Auburn, Alabama, and kept at refrigeration temperatures ( $\sim 4$  °C) until processing within 5 h of purchase. Samples were grouped as breast tenders, boneless breasts, split breasts, thighs (including boneless thighs), and skin from breast and thighs. Samples were collected throughout 13 collection times.

### 2.2. Sample preparation and enrichment procedures

From each tray pack, one piece of meat was sampled by weighing 25 g of product twice (two sub-samples). The samples were placed in sterile Whirl-Pak® bags (Nasco, Fort Atkinson, WI). One sub-sample was enriched at a 1:9 ratio (w:v), where 25 g was dissolved in 225 ml of buffered peptone water (BPW, Acumedia, Baltimore, MD) supplemented with 5% lysed horse blood (School of Veterinary Medicine, Auburn University, Institutional Animal Care Committee Protocol PRN 2004–0623) and 2 mg/l of amphotericin B, 32 mg/l of cefoperazone, 10 mg/l of trimethoprim, and 10 mg/l of vancomycin (Sigma-Aldrich, St. Louis, MO). In parallel, the other sub-sample was enriched in Bolton broth following the specifications of the manufacturer (Oxoid Inc., New York, NY). Briefly, the Bolton basal medium (CM0983) was combined with Bolton broth selective supplements (SR183) and with 5% lysed horse blood. These sub-samples were enriched at a 1:9 ratio (w:v). Enriched samples were incubated at 42 °C for 24 h under a microaerophilic gas mixture containing 10% CO<sub>2</sub>, 5% O<sub>2</sub>, and 85% N<sub>2</sub> (Airgas, Radnor, PA) in sealed, plastic bags (Oyarzabal et al., 2005), or in anaerobic jars gassed with a MACSmics Jar Gassing System (Microbiology International, Frederick, MD).

### 2.3. *Campylobacter* isolation and presumptive identification

All enriched samples were plated out for isolation and identification of *Campylobacter* spp. A sterile cotton swab (Fisher Scientific, Pittsburgh, PA) was used to transfer  $\sim 0.1$  ml of the enrichment broth onto modified Campy-Cefex (mCC) agar (Oyarzabal et al., 2005; Stern et al., 1992) plates. Plates were then incubated at 42 °C under microaerophilic conditions for 48 h and then screened for typical *Campylobacter* colonies. Colonies were considered presumptive positive when they showed the typical morphology and motility under phase contrast microscopy (Optiphot-2, Nikon, Tokyo, Japan) and when they were catalase and oxidase positive. All presumptive isolates were collected and individually stored at  $-80$  °C in tryptic soy broth (TSB, Difco, Detroit, MI) supplemented with 30% glycerol (vol/vol) and 5% blood. These isolates were used for further identification with a multiplex PCR assay and characterization by PFGE.

### 2.4. PCR identification of *Campylobacter* isolates

For PCR analysis, stock cultures were transferred to mCC plates. Plates were incubated under microaerophilic conditions at 42 °C for 24 h. Bacterial DNA was extracted using PrepMan™ Ultra (Applied Biosystems, Foster City, CA). All the DNA was tested with a multiplex PCR that has been used for the identification of *C. jejuni* and *C. coli* from swine samples (Cloak and Fratamico, 2002) and processed broilers carcasses (Oyarzabal et al., 2005). With this multiplex PCR, isolates that react with primers targeting the *ceuE* gene are *C. coli* (Gonzalez et al., 1997), while those isolates that react with primers targeting an undefined portion of DNA from the genome of *C. jejuni* are

considered *C. jejuni* (Winters and Slavik, 1995). DNA samples were also tested with a second multiplex PCR assay that is based on three sets of primers targeting the aspartokinase gene (forward primer CC18F, 5'-GGT ATG ATT TCT ACA AAG CGA G-3'; reverse primer CC519R, 5'-ATA AAA GAC TAT CGT CGC GTG-3'; Linton et al., 1997), the hippuricase gene (forward primer HipO-F, 5'-GAC TTC GTG CAG ATA TGG ATG CTT-3'; reverse primer HipO-R, 5'-GCT ATA ACT ATC CGA AGA AGC CAT CA-3') and the 16S rDNA genes (forward primer 16S-F, 5'-GGA GGC AGC AGT AGG GAA TA-3'; reverse primer 16S-R 5'-TGA CGG GCG GTG AGT ACA AG-3'). Primers for the hippuricase and 16S rDNA genes have been published by Persson and Olsen (2005). PCR assays were performed in 25  $\mu$ l aliquots, with 18  $\mu$ l of a pre-made mix (OmniMix<sup>®</sup> HS, Cepheid, Sunnyvale, CA), 1  $\mu$ l of each primer (10  $\mu$ M) and 1  $\mu$ l of the DNA template (~50 ng). The assay was performed in a PTC-100 Programmable Thermal Controller (MJ Research, Inc.). A temperature of 94 °C for 4 min was used to denature DNA, followed by 20 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min. Final extension was done at 72 °C for 5 min. Amplicons were detected by standard gel electrophoresis in 1.5% agarose (Ultra Pure DNA Grade Agarose, Bio-Rad Laboratories, Hercules, CA, USA) in Tris-borate–EDTA buffer at 70 V for 1 h. DNA bands in the gels were stained with ethidium bromide and visualized using a UV transilluminator (Gel-Doc System) with a Molecular Analyst computer program (Bio-Rad Laboratories).

### 2.5. Sequencing of amplicons

Amplicons from *C. jejuni* and *C. coli* strains were sequenced at the Auburn University Genomics & Sequencing Lab using an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). The 1062-bp amplicon corresponding to the 16S DNA genes were also amplified. In addition, the 16S genes from selected *C. jejuni* and *C. coli* strains were sequenced using the primers described by Burnett et al. (2002). Sequences were compared for similarity using the nucleotide–nucleotide search feature of BLAST (<http://ncbi.nih.gov/BLAST/>).

### 2.6. Assessment of 1:4 enrichment ratio in BPW for *Campylobacter* isolation

For 63 samples, an extra sub-sample of 25 g of meat was collected to determine the efficacy of adding only 100 ml of BPW (1:4 ratio) for enrichment. These samples were enriched for 24 h under a microaerophilic conditions at 42 °C. *Campylobacter* isolation and presumptive identification was performed as described under 2.3. These results were compared with the results obtained from the same samples enriched using a 1:9 ratio.

### 2.7. Most probable number procedure for enumeration of *Campylobacter* spp.

For 59 samples, a 3-tube most probable number (MPN; 10 g, 1 g, 0.1 g) procedure was performed with Bolton and BPW (1:9 ratio) to determine if BPW performs similarly as Bolton broth

for MPN enumeration of *Campylobacter* spp. Samples were enriched for 24 h under microaerophilic conditions and transferred to mCC plates for isolation. Identification of positive samples was done by the observation of typical morphology and motility under phase contrast microscopy. The calculation of the MPN values was done using the updated MPN tables published by the U. S. Department of Agriculture Food Safety and Inspection Services (Anonymous, 2003). For statistical analysis, samples with MPN results of 0.0 were given a value of 0.02 CFU/g.

### 2.8. PFGE analysis of isolates from the same samples

A standard PFGE protocol with the modifications suggested by the Centers for Disease Control and Preventions was used (Available from URL <http://www.cdc.gov/pulsenet/protocols.htm>; Ribot et al., 2001). A lambda DNA size marker (48.5 kbp and its oligomers, Promega, Madison, WI) was included in all PFGE gels. *Campylobacter* DNA was digested with *Sma*I and analyzed with a contour-clamped homogeneous electric field (CHEF, Bio-Rad, Hercules, CA) in 1% agarose gel, then stained with ethidium bromide and visualized with a UV transilluminator (Gel-Doc System, Bio-Rad). Pair comparison and cluster analysis was done using the Dice correlation coefficient and the unweighted pair group mathematical average (UPGMA) clustering algorithm of BioNumerics version 4.50 (Applied Maths, Austin, TX). The optimization and position tolerance for band analysis were set at 1%. A cutoff of 90% was used for the determination of the different PFGE patterns (De Boer et al., 2000).

### 2.9. Statistical analysis

Results from the enrichment were compared in the following way: Bolton broth and BPW; BPW 1:9 and BPW 1:4; and Bolton broth 1:9 and BPW 1:4. The analysis of these comparisons was done using chi-square ( $\chi^2$ ) according to McNemar (Feldsine et al., 2000), where a chi-square value  $\leq 3.84$  indicated that the hypothesis that the test method (BPW) and reference method (Bolton broth) are equivalent and could not be rejected at the 5% level of confidence. The formula for this calculation is:

$$\chi^2 = \frac{(|a-b|-1)^2}{(a+b)}$$

Where  $a$ =test samples positive in BPW and negative in Bolton broth;  $b$ =test samples negative in BPW and positive in Bolton broth.

The total number of positives obtained with Bolton broth, BPW and both enrichments were analyzed for differences using the chi-square calculated with the non-parametric, Kruskal–Wallis test (NPAR1WAY procedure) of SAS (SAS Release 9.1, SAS Institute Incorporated, Cary, NC), with significant set at  $\leq 3.84$ . MPN data were analyzed using the Univariate procedure of SAS for descriptive statistics and goodness-of-fit tests for normal distribution. A histogram of distribution

Table 1  
*Campylobacter* spp. from retail broiler samples

Product	No. of samples	Negative	Positive (%)			Positive in BPW negative in Bolton (A) <sup>a</sup>	Negative in BPW positive in Bolton (B) <sup>a</sup>	Positive in BPW and Bolton	$\chi^2$
			Total	Bolton	BPW				
Tenders	22	14	8 (36)	6 (27)	6 (27)	2	2	4	0.25
Boneless breasts	25	5	20 (80)	17 (68)	17 (68)	3	3	14	0.17
Split breasts	24	11	13 (54)	6 (25)	11 (46)	7	2	4	1.78
Thighs (including boneless thighs)	25	5	20 (80)	19 (76)	12 (48)	1	8	11	7.11
Skin <sup>b</sup>	17	4	13 (76)	11 (65)	11 (65)	2	2	9	0.25
Total	113	39	74 (65)	59 (52)	57 (50)	15	17	42	0.28

Comparison of results between positive samples in Bolton broth and in BPW.

<sup>a</sup> Chi-square calculation based on column A and B.

<sup>b</sup> Skin from breast and thighs.

(Excel, Microsoft, Redmond, WA) showed that the MPN data did not fit into a normal distribution. Hence, the Wilcoxon Two-Sample test (NPAR1WAY procedure of SAS) was used to compare the MPN results from BPW and Bolton broths for differences.

### 3. Results

#### 3.1. Efficacy of buffered peptone water to isolate *Campylobacter* spp.

From 113 broiler samples enriched for *Campylobacter* spp., 74 samples (65%) were found positive. Bolton broth yielded 52% positive samples, while BPW yielded 50% positive samples (Table 1). A total of 37 samples were positive in both sub-samples (Bolton broth and BPW). Chi-square analysis showed that BPW was similar to Bolton broth for *Campylobacter* isolation in breast tenders, boneless breasts, split breasts and skin samples ( $\chi^2=0.25$ , 0.17 and 1.78, respectively). However, BPW showed a lower detection rate for thighs and boneless thighs ( $\chi^2=7.11$ ). When combining these results, BPW was similar to Bolton broth for the isolation of *Campylobacter* spp. from broiler retail samples ( $\chi^2=0.28$ ).

#### 3.2. PCR identification of *Campylobacter* spp. Isolates

Initially, the second multiplex PCR assay was validated with ATCC (American Type Culture Collection) strains of *C. jejuni* (ATCC 700819, 35918, and 33560), *C. coli* (ATCC 43473 and BAA-371), *C. fetus* (ATCC 27374), *C. hyointestinalis* (ATCC 35217), *C. lari* (ATCC 43675 and 35223), and *C. upsiliensis* (ATCC 43953). All ATCC strains yielded the expected 1062-bp amplicon for the 16S rDNA gene, but only *C. jejuni* strains yielded the expected amplicon for the hippurate gene (344 bp) and only *C. coli* strains yielded the expected amplicon for the aspartate kinase gene (500 bp) (data not shown).

The isolates from 69 samples (103 isolates, 54 from Bolton broth and 49 from BPW) were tested with the multiplex PCR assay. Forty-seven percent of these isolates were *C. jejuni* while 53% of the isolates were *C. coli*. Forty-eight percent of the isolates obtained from Bolton broth were *C. coli*, while 59% of the isolates obtained with BPW were *C. coli* (Table 2). These 69

samples were also tested with a multiplex PCR that has been used for the identification of *C. jejuni* and *C. coli* from swine samples (Cloak and Fratamico, 2002) and processed broilers carcasses (Oyarzabal et al., 2005). With this multiplex PCR, isolates that react with primers targeting the *ceuE* gene are *C. coli* (Gonzalez et al., 1997), while those isolates that react with primers targeting an undefined portion of DNA from the genome of *C. jejuni* are considered *C. jejuni* (Winters and Slavik, 1995). Results from these two multiplex PCR were in complete agreement.

In five samples, the isolates from Bolton broth were *C. coli* while the isolates from BPW were *C. jejuni*. In five samples, isolates from Bolton broth were *C. jejuni* while the isolates from BPW were *C. coli*. Six sub-samples, representing five different samples, were contaminated with both *C. jejuni* and *C. coli*, and gave the expected amplicons with PCR analysis (data not shown). We have found that using a 0.45 or a 0.6  $\mu\text{m}$  sterile cellulose acetate membrane filter on agar plates helps obtain isolated colonies for further studies (Baggerman and Koster, 1992; Baggerman and Vervoort 1987; Steele and McDermott 1983). Further purification of the mixed isolates resulted in three *C. coli* and three *C. jejuni* isolates (Fig. 1-A).

#### 3.3. Sequencing results

The BLAST search of the 500-bp amplicon specific for *C. coli* gave a 100% similarity to the aspartate kinase gene from *C. coli* strain NCTC 11366 (accession number AF017758), but

Table 2  
 Results from the analysis of 103 isolates with a multiplex PCR for identification of *C. jejuni* and *C. coli*<sup>a</sup>

Product	Number of isolates	Bolton		BPW	
		<i>C. jejuni</i>	<i>C. coli</i>	<i>C. jejuni</i>	<i>C. coli</i>
Tenders	11	5	1	3	2
Boneless breasts	30	6	8	7	9
Split breasts	15	4	2	5	4
Thighs (including boneless thighs)	28	9	8	4	7
Skin	19	4	7	1	7
Total	103	28	26	20	29

<sup>a</sup> *C. coli* isolates were also confirmed with another multiplex PCR (Cloak and Fratamico, 2002; Oyarzabal et al., 2005).

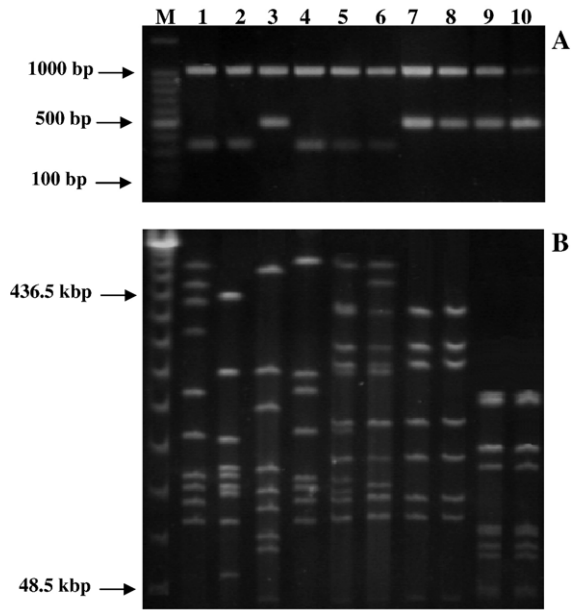


Fig. 1. PCR (A) and PFGE (B) results of *Campylobacter* isolates from samples that were positive in Bolton broth and BPW. M: 100-bp molecular marker for PCR and 48.5-kbp lambda marker for PFGE. Strains with 500-bp and 1062-bp amplicons are *C. coli*, while strains with 344-bp and 1062-bp amplicons are *C. jejuni*. Lane designations: 1 = A-Bolton (*C. jejuni*); 2 = A-BPW (*C. jejuni*); 3 = B-Bolton (*C. coli*); 4 = B-BPW (*C. jejuni*); 5 = C-Bolton (*C. jejuni*); 6 = C-BPW (*C. jejuni*); 7 = D-Bolton (*C. coli*); 8 = D-BPW (*C. coli*); 9 = E-Bolton (*C. coli*); 10 = E-BPW (*C. coli*).

only 86% similarity to the aspartate kinase gene from *C. jejuni* strain NCTC 11168 (accession number AL139075). The search of the sequencing of the 344-bp amplicon from the hippurate gene gave a 96% similarity to *C. jejuni* (accession number AY944174). The alignment of the sequences from the 1062-bp amplicons from *C. jejuni* and *C. coli* gave a 98% identity between these sequences, and the BLAST search also revealed a sequence similarity of 98% with *C. jejuni* and *C. coli*. The search with the sequencing from the 16S DNA from *C. jejuni* and *C. coli* corroborated the identity of the strains.

#### 3.4. Enrichment ratio comparison

Table 3 shows the results from the comparison of BPW at 1:4 ratio (25 g in 100 ml) versus Bolton broth or BPW at a 1:9 ratio (25 g in 225 ml). BPW at 1:4 ratio was similar to Bolton broth or BPW at 1:9 ratios for the detection of *Campylobacter* spp. ( $\chi^2 \leq 1.79$ ).

Table 3  
Comparison of the results from 1:4 and 1:9 enrichment ratios

No. of samples	Positive			Positive in 100 BPW, negative in (A) <sup>a</sup>		Negative in 100 BPW, positive in Bolton or BPW (B) <sup>a</sup>	Total		$\chi^2$ comparison	
	Bolton	BPW	100 ml BPW	Bolton	BPW		Negative	Positive	Bolton	BPW
63	28	29	25	6	5	9	29	34	1.07	1.79

Comparison was made between BPW 1:4 (25 g in 100 ml) and Bolton broth 1:9 (25 g in 225 ml) or BPW 1:9 (25 g in 25 ml).

<sup>a</sup> Chi-square calculations were based on column A and B.

Table 4

Percentage similarity of isolates from the same samples obtained in Bolton broth and BPW with PFGE

Isolate ID		Sample <sup>a</sup>	% Similarity
Bolton broth	BPW		
<i>C. jejuni</i>	<i>C. jejuni</i>	G	99.9
		C	86.9
		I	57.1
		A	52.6
		Q	35.3
		J	27.2
<i>C. coli</i>	<i>C. coli</i>	D, E, M, N, P, R, T, U, V and X	99.9
		F	70.5
		L	82.3
		S	55.5
		W	47.0
		Y	53.3

Pair-comparison of 50 isolates from 25 samples. The analysis was performed with the Dice correlation coefficient and UPGMA clustering algorithm of BioNumerics.

<sup>a</sup> Three samples (H, K and O) had *C. jejuni* in Bolton and *C. coli* in BPW. Sample (B) had *C. coli* in Bolton and *C. jejuni* in BPW. Isolates from Bolton broth and BPW for samples G, M, R, U and V were also restricted with *KpnI*. PFGE of *KpnI* macrorestriction also exhibited a 99.9% similarity between isolates from the same samples.

#### 3.5. MPN values from Bolton broth and BPW

A histogram of the MPN values showed that the data were not normally distributed. This finding was also confirmed with a comparison of each data set using the Kolmogorov–Smirnov test. Therefore, we decided to analyze the raw data using the Wilcoxon matched-pairs signed-ranks test. No differences ( $P \leq 0.50$ ) were observed between the mean MPN data from Bolton broth (0.64 CFU/g) and the mean MPN data for BPW (0.79 CFU/g) from 65 samples (130 sub-samples) analyzed with the MPN method. Seven samples enriched with Bolton broth and eight samples enriched with BPW had counts between 1 and 5 CFU/ml. One sample enriched in Bolton broth had a count of 12 CFU/g and one sample enriched in BPW had a count of 11 CFU/g.

#### 3.6. PFGE results

PFGE was performed on a total of 50 isolates (34 *C. coli* and 16 *C. jejuni* strains) obtained from 25 samples (termed A through Y) that were positive in both Bolton and BPW in order to determine genomic similarities. The reproducibility of the

PFGE results was verified by analyzing 12 isolates (five *C. jejuni* and seven *C. coli*) in two separate experiments. The reproducibility was more than 99% for all 12 isolates. Therefore, we considered appropriate to adopt a cutoff value of 90% for the percentage similarity analysis. With this cutoff value, there were 13 different *C. jejuni* PFGE profiles and 18 different *C. coli* PFGE profiles (Fig. 1 B). The number of bands in the PFGE patterns ranged from seven to 15 (Fig. 1 B). Pair comparison using Dice correlation coefficient and UPGMA clustering algorithm of BioNumerics showed a large variation in the DNA relatedness of samples containing *C. jejuni* in Bolton broth and BPW. *C. coli* isolates showed less variability than *C. jejuni* isolates, although a sample exhibited only 47% similarity between the *C. coli* isolates from Bolton broth and BPW (Table 4). Unlike previous reports (De Boer et al., 2000; Yan et al., 1991), we were not able to discriminate among *C. jejuni* and *C. coli* isolates based on PFGE restriction patterns.

#### 4. Discussion

The recovery of thermotolerant *Campylobacter* spp. in retail samples is problematic and direct plating has failed to detect *Campylobacter* in retail samples (Willis and Murray, 1997). Enrichment procedures are still the gold-standard methodology to determine if a food sample is positive for *Campylobacter* spp. or not. However, the concentration of bacteria by centrifugation or filtration and centrifugation have been used to increase the number of *Campylobacter* cells before plating and to by-pass the enrichment step (Dickins et al., 2002; Nannapaneni et al., 2005; Willis and Murray, 1997). If BPW could be used as an enrichment procedure, a standard method for *Campylobacter* testing could be developed for processed carcasses as well as retail products. Here, we studied the efficacy of BPW supplemented with lysed horse blood and antibiotics for the isolation of thermotolerant *Campylobacter* spp. from broiler retail products. We used Bolton broth as the standard broth for comparison because this medium that has been used most frequently for isolation of *Campylobacter* from poultry samples (Anonymous, 1998; Paulsen et al., 2005), and it appears to be one of the best available alternatives to compromise between the inhibition of competitors and the growth of *Campylobacter* spp. (Baylis et al., 2000).

The number of positive samples found in this study correlated with previous publications from the US (Dickins et al., 2002; Willis and Murray, 1997) and Europe (Atanassova and Ring, 1999; Dufrenne et al., 2001; Kramer et al., 2000; Scherer et al., 2006). Although the analysis of all samples showed that BPW could be used as an alternative to isolate *Campylobacter* from retail samples, more research is necessary to determine the efficacy of BPW to isolate *Campylobacter* from thighs and legs. These findings suggest that the composition and quality of the meat may play an important role on the efficacy of supplemented BPW, and perhaps other enrichment broths, to isolate *Campylobacter* from broiler meat. In addition, an increase in more than 10% in the number of positive samples was found when the results from Bolton and BPW were combined, which highlights that the analysis of

more than 25 g for sample will increase the chances of detecting *Campylobacter* positive samples.

The MPN comparison with Bolton broth and BPW also helped validate BPW as a potential enrichment broth for use when isolating *Campylobacter* spp. The fact that samples which were negative by enriching only 25 g of product gave positive results after the MPN analysis suggests again that a larger food sample size should be used for standard analyses. The number of *Campylobacter* as CFU/g calculated with the MPN was less than 1 in 85% of the samples. Therefore, the actual number of CFU contaminating retail broiler products is very low and can only be detected with concentration methods or enrichment procedures.

We also wanted to know if the addition of only four times the volume of enrichment to the sample weight has any influence in the isolation rate for *Campylobacter* in retail samples. For *Listeria*, it has been suggested that a 1:4 enrichment ratio performs similarly to the tradition 1:9 ratio, but greatly reduces the cost when processing a high number of samples (Stevens et al., 2005). Our results with a panel of 63 samples showed that a 1:4 enrichment protocol is more efficient for the isolation of *Campylobacter* from broiler samples. Such a protocol would allow for an increase in the sample size to twice of what is used currently (25 g) with an actual decrease in the volume of enrichment necessary to analyze the sample. Ultimately, an increase in the sample size will yield more realistic results for risk assessment studies. Simulation results have shown that the incidence and distribution of *Salmonella* contamination in broiler meat increases in a non-linear manner as a function of sample size, with a 16% estimated incidence in 25-g samples and a 51% estimated incidence in 100-g samples (Oscar, 2004). Thus, linear extrapolation of enumeration results, a common practice in microbial risk assessment, may not appear to be appropriate (Oscar, 2004). Presently, we do not know the most appropriate samples size for *Campylobacter* identification and risk analysis purposes.

We believe these are the first results showing that more than one *Campylobacter* isolate, and even two species, *C. jejuni* and *C. coli*, can be detected from the same retail sample after enrichment if all the growth of the plate is kept for analysis. Our PCR results were confirmed by using two multiplex PCR, and the sequencing of the amplicons of the multiplex PCR described in this paper. Traditionally, only few similar colonies from agar plates are kept for further identification. However, the spread, undefined growth that *Campylobacter* exhibits on mCC agar plates makes it difficult to collect isolated colonies. Considering that only 0.1 ml from the enriched broth was transferred onto an agar plate to determine if a sub-sample was positive or negative, the chances for underestimating the actual number of positive samples was high.

The importance of identifying the isolates to the species level must also be highlighted. Our results demonstrated that a variety of *C. jejuni* and *C. coli* strains that contaminate retail samples could be isolated, in part, using enrichment procedures. Conversely, we do not know the extent of inhibition that enrichment broth can exert on specific *C. jejuni* and *C. coli* genotypes. The extent of this genome variation was demonstrated by the

diversity of the PFGE profiles from *C. jejuni* and *C. coli* isolates obtained from the same samples. The coexistence of different isolates and even two species has been determined by direct plating, after concentration procedures, but it has not yet been reported from enriched samples. These findings highlight the challenges of obtaining single, isolated colonies on agar plates from swarming bacteria, such as *C. jejuni* and *C. coli*, for PFGE analysis (Barrett et al., 2006). In these cases, again, a filtration system may be useful for identification of single colonies on plates.

Another surprising finding of our study was that 55% of the isolates tested with the multiplex PCR were *C. coli*. All *C. coli* isolates were confirmed twice using two multiplex PCR, one targeting the aspartate kinase gene and the other targeting the *ceuE* gene of *C. coli*. The primers targeting the aspartate kinase gene have been found to be the most suitable primers for *C. coli* identification from several reported PCR protocols (On and Jordan, 2003). Whether these findings are representative of a unique area or seasonal conditions, or reflect a lower adaptation to survival by *C. coli*, is still debatable. Perhaps the direct plating technique used consistently for *Campylobacter* detection on broiler carcasses in the US may be inhibitory to *C. coli* and may bias the results towards a higher number of *C. jejuni*. The composition of the enrichment media may also play a role on selecting some species or strain more than others, as it appears to be the case with the higher number of *C. coli* observed in sub-samples enriched in BPW.

The variability of the PFGE patterns of isolates obtained from Bolton broth and BPW and from the same sample was somewhat surprising. The large percentage of variability based on the Dice correlation coefficient and UPGMA clustering algorithm support the idea that the enrichment procedure allow for more than one genotype to successfully multiply and be recoverable. Our results suggest that PFGE analysis of *Campylobacter* isolates from broiler samples yield patterns that are not discriminatory between *C. jejuni* and *C. coli*. None of the fingerprinting methods appears to be an ideal method for discrimination of *C. jejuni* and *C. coli* strains. However, PFGE still remains as the gold standard for epidemiological analysis and is highly reproducible. PFGE has also been shown to be more discriminatory than other fingerprinting procedures when investigating the variations in the fingerprinting pattern of isolates infecting humans (Steinbrueckner et al., 2001).

In summary, the results showed that BPW performed similarly as Bolton broth for *Campylobacter* isolation from broiler products; BPW at an enrichment ratio of 1:4 was statistically similar to Bolton broth or BPW at a ratio of 1:9 for *Campylobacter* isolation; there were no differences between the MPN data from Bolton broth and the MPN data from BPW; a large number of *C. coli* isolates were identified from both enrichment broths; and more than one genotype can successfully multiply during enrichment and be recoverable on agar plates.

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