Research Note: A baseline survey of thermotolerant *Campylobacter* in retail chicken in southern Brazil

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ABSTRACT Chicken is a leading source of thermotolerant *Campylobacter*, which triggers human foodborne enteritis. This study evaluated thermotolerant *Campylobacter* contamination of retail chicken in southern Brazil, using qualitative and quantitative analyses. Selective enrichment in Bolton broth for 24 and 48 h after plating onto modified charcoal–cefoperazone–deoxycholate (**mCCD**) agar and Preston agar was assessed. The combined results of the detection and enumeration methods revealed a frequency of 70% occurrence of thermotolerant *Campylobacter* in chicken samples. *Campylobacter* was enumerated in 60% of the samples, whereas 46% of the samples were positive in the qualitative analysis. Quantitative analysis showed average counts of 3.10 ± 0.15 $\log_{10}$ CFU/sample. Higher numbers of *Campylobacter*-positive samples were found using 24-h enrichment before plating onto Preston agar (46%) than onto mCCD agar (2%). The majority of isolated strains were identified as *Campylobacter jejuni*, and *Campylobacter coli* was also found but to a lesser extent. Subtyping revealed a clear distinction between strains isolated from different chicken sources. The enriched samples plated onto mCCD agar showed extensive spreading of nonproducing extended-spectrum β-lactamases *Proteus mirabilis* that hampered the identification of *Campylobacter* colonies. *P. mirabilis* strains showed resistance to cefoperazone, trimethoprim, and polymyxin B present in broth and plate media used and were inhibited by rifampicin present in Preston agar. The results underline the effect of the spread of contaminant strains on *Campylobacter* cultures, which might be prevented using a recently revised International Organization for Standardization method for qualitative analysis of chicken.

Key words: *Campylobacter*, poultry meat, microbial contamination, isolation method

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INTRODUCTION

Campylobacteriosis is among the leading foodborne diseases reported worldwide (Rossler et al., 2019; Tack et al., 2019). *Campylobacter jejuni* accounts for most of the 90% of culture-confirmed human cases, whereas infection with *Campylobacter coli* and other related species occurs to a lesser extent (Patrick et al., 2018). Various foods are potential sources of *Campylobacter*, but the consumption of mishandled or undercooked chicken is the main risk factor for foodborne campylobacteriosis (Rossler et al., 2019). Quantitative risk assessment has revealed that the incidence of campylobacteriosis associated with consumption of chicken could be reduced 30 times by introducing a 2-log reduction of the *Campylobacter* population on the carcasses (Rosenquist et al., 2003). Particularly, proper food safety practices and operations at broiler processing are crucial for reducing exposure to *Campylobacter* through chicken consumption (Dogan et al., 2019).

The number of *Campylobacter* per sample of raw chicken represents a critical measure of human exposure to the bacterium. In 2017, the European Union laid down a process hygiene criterion for thermotolerant *Campylobacter* not to exceed 1,000 CFU/g in neck skin taken from a given number of broiler carcasses upon a defined sampling plan at slaughter (European Council, 2017). Meanwhile, surveillance of *Campylobacter* in chicken at retail is crucial to assessing the associated potential risk for human campylobacteriosis and offering opportunities to improve strategies for consumer education (Stella et al., 2017; Habib et al., 2019).

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The isolation of *Campylobacter* from foods is difficult because of the fastidious growth requirements and the competing microbiota (Moran et al., 2011). Most selective broth and plate media available to isolate *Campylobacter* contain several antimicrobials as the primary inhibitor of contaminant microorganisms (Gharst et al., 2013). An enrichment step is essential to analyze samples in which low numbers of *Campylobacter* cells are expected (Sproston et al., 2014), yet extending the enrichment time might promote the growth of less fastidious antimicrobial-resistant bacteria (Jasson et al., 2009). Moreover, the emergence of resistant nontarget bacteria, such as extended-spectrum β-lactamases (**ESBLs**)–producing *Escherichia coli*, means the detection of *Campylobacter* using selective broth containing β-lactams might lead to underestimating its prevalence in chicken samples (Jasson et al., 2009; Chon et al., 2017; Biesta-Peters et al., 2019). This study used both qualitative and quantitative bacteriological analyses to evaluate thermotolerant *Campylobacter* contamination in retail chicken in southern Brazil. To optimize the isolation of *Campylobacter*, samples were concurrently enriched for 24 and 48 h, and contaminant strains of *Campylobacter* cultures were also determined.

MATERIALS AND METHODS

Sampling

Fifty separate tray packs of fresh chicken pieces with skin (breasts, leg thighs, and drumsticks) and an average weight of 753.24 ± 116.95 g, sourced from 2 processors in southern Brazil, were obtained at local retail markets between 2013 and 2015. All samples were portioned and packed by the retail market and kept on chilled shelves. Samples were transported to the laboratory in insulated boxes with ice packs and processed within 1 h. At the laboratory, the initial suspension was prepared by hand rinsing the samples placed in a sterile polyethylene bag with 150 mL of 0.1% buffered peptone water for 1 min.

Qualitative Analysis

Thermotolerant *Campylobacter* species were detected, as previously described (International Organization for Standardization [ISO], 2006a). Accordingly, 10 mL of the initial suspension was added to 90 mL of Bolton broth (Oxoid, Basingstoke, Hampshire, UK) supplemented with 5% lysed horse blood, 20 μg/mL of cefoperazone, 20 μg/mL of vancomycin, 20 μg/mL of trimethoprim lactate, and 10 μg/mL of amphotericin B (Sigma–Aldrich, St. Louis, MO). Samples were homogenized and incubated in a microaerobic atmosphere (5% O_2, 10% CO_2, with balanced N_2; White Martins, Rio de Janeiro, Brazil) at 37°C for 4 to 6 h and then at 41.5°C for 48 h. A minor method modification included the concurrent enrichment of the samples for 24 h. Enriched samples were plated onto modified charcoal–cefoperazone–deoxycholate (**mCCD**) agar (Oxoid) supplemented with 32 μg/mL of cefoperazone and 10 μg/mL of amphotericin B (Sigma–Aldrich). Preston agar (Oxoid) supplemented with 0.6 μg/mL (5 IU/mL) of polymyxin B, 10 μg/mL of rifampicin, 10 μg/mL of trimethoprim lactate, and 10 μg/mL of amphotericin B (Sigma–Aldrich) was used as the second selective medium. Plates were incubated at 41.5°C for 44 ± 4 h. After microscopy, at least one typical colony was taken from each selective agar and subcultured onto blood agar no. 2 (Oxoid) to further analyze for oxidase, sodium hippurate, and indoxyl acetate, as described (ISO, 2006a).

Quantitative Analysis

Thermotolerant *Campylobacter* quantitative analysis was conducted using 1 mL of the initial suspension, which was distributed on the surface of 3 mCCD agar plates (90 mm), as per ISO 10272-2:2006 (ISO, 2006b). Five randomly selected colonies from each selected plate were subcultured onto blood agar no. 2 for the phenotypic confirmation, as described in the qualitative analysis. The *Campylobacter* counts were converted into $\log_{10}$ (CFU/sample) and categorized as follows: <150 CFU/sample (undetectable); 2 to 3 $\log_{10}$ CFU/sample; >3 to 4 $\log_{10}$ CFU/sample; >4 $\log_{10}$ CFU/sample.

DNA MacroRestriction Analysis

One *Campylobacter* isolate, phenotypically characterized from each positive chicken sample, as detected by qualitative or quantitative analysis in either Preston agar or mCCD agar, was randomly selected for subtyping, as described (Ribot et al., 2001). *Sma*I (New England Biolabs, Hitchin, Hertfordshire, UK)-digested DNA was separated by pulsed-field gel electrophoresis (**PFGE**), using initial and final switch times of 6.75 and 38.35 s, respectively, in a CHEF Mapper XA system (Bio-Rad, Hercules, CA) on a 1.4% agarose PFGE-certified gel (Bio-Rad) for 23 h at 6.6 V/cm and a 120° angle. *Xba*I (New England Biolabs)-digested DNA of *Salmonella* Braenderup strain H9812 (ATCC BAA-664) was used as the size standard. DNA macrorestriction patterns were analyzed using BioNumerics 6.1 (Applied Maths, Sint-Martens-Latem, East Flanders, Belgium). The Dice coefficient (1.5% position tolerance) was used to calculate similarity. A dendrogram was generated by cluster analysis using the unweighted paired group method with arithmetic average.

Characterization of Contaminant Strains

Twenty-three contaminant strains from different analyzed samples that overgrew *Campylobacter* cultures on mCCD agar plates in the qualitative analysis were randomly selected, Gram-stained, and subcultured in brain heart infusion agar (BD Difco, Detroit, MI) and MacConkey agar (Merck, Darmstadt, Hesse, Germany) at 37°C for 24 h. Then, isolated strains were characterized by standard biochemical procedures (Holt et al., 1994). The minimum inhibitory concentrations (**MICs**)

to cefoperazone, trimethoprim lactate, polymyxin B, and rifampicin were determined by the standard broth dilution procedure using Mueller–Hinton broth (BD Difco) (Clinical and Laboratory Standards Institute [CLSI], 2015a). *E. coli* ATCC 25922 and *Enterococcus faecalis* ATCC 29212 were used to monitor the accuracy of the MICs. The isolated contaminant strains were screened and confirmed for ESBL production, by the disc diffusion method, using 10 μg ceftazidime, 30 μg cefotaxime, 30 μg ceftazidime–10 μg clavulanic acid, and 30 μg cefotaxime–10 μg clavulanic acid (BD Difco), as described (CLSI, 2015b). *Klebsiella pneumoniae* ATCC 700603 and *E. coli* ATCC 25922 were used as quality controls.

Statistical Analysis

The effect of enrichment in Bolton broth for 24 and 48 h and plating onto mCCD agar and Preston agar was analyzed by Fisher's exact test. The efficacy of mCCD agar for *Campylobacter* enumeration, qualitative detection of positive samples, and the qualitative prevalence of *Campylobacter* species were compared using the chi-square test (χ^2). All statistical analyses were accomplished using SAS, version 9.4, (SAS Institute, Inc., Cary, NC) at $P \leq 0.05$.

RESULTS AND DISCUSSION

Based on the combined detection and enumeration data, 35 of 50 (70%) analyzed chicken samples were contaminated with thermotolerant *Campylobacter*. Qualitative analysis indicated that 23 of 50 (46%) samples were *Campylobacter*-positive compared with 30 of 50 (60%) by the quantitative method. *Campylobacter* was enumerated in 12 samples that were negative in the qualitative analysis, whereas 5 *Campylobacter*-positive samples showed quantitatively undetectable counts (Figure 1). These results confirmed that a combination of both detection and enumeration methods to determine prevalence increases the probability of obtaining positive test results for *Campylobacter* in broiler carcasses than a single examination by either method (European Food Safety Authority [EFSA], 2010).

Other studies have reported *Campylobacter* contamination rates in poultry meat of between 34.1 to 58.8% (Giombelli and Gloria, 2014; Panzenhagen et al., 2016; Seliwiorstow et al., 2016; Stella et al., 2017; Habib et al., 2019). *Campylobacter* contamination reduction is usually expected throughout broiler production steps, from the farm to consumer (Dogan et al., 2019). Interestingly, the combined 70% frequency of *Campylobacter* found in retail chicken in this study was higher than the previous 45.0% (Panzenhagen et al., 2016) to 58.8% (Giombelli and Gloria, 2014) reported on carcasses sampled at Brazilian broiler slaughterhouses. Nonetheless, differences between laboratory procedures, other than ISO, as used here, hinder a direct comparison of the results.

Quantitative analysis indicated an average of 3.10 ± 0.15 $\log_{10}$ CFU/sample. Notably, chicken samples with the highest levels of *Campylobacter* were more frequently detected as positive in the qualitative analysis (Figure 1). Most samples contained *Campylobacter* levels similar to those described in broiler carcasses sampled at the postchilling stage in the USA, of 2.50 $\log_{10}$ CFU/carcass (Dogan et al., 2019). As previously reported, low *Campylobacter* counts have been detected in chicken samples judged to be qualitatively positive (Stella et al., 2017; Habib et al., 2019). However, independent of the *Campylobacter* dose ingested from contaminated chicken in Denmark, Rosenquist et al. (2003) estimated a 22% probability of a person becoming ill once infected.

mCCD agar showed greater efficacy to enumerate *Campylobacter* than to isolate the bacterium after 24-h enrichment in Bolton broth ($P < 0.0001$) in concurrently analyzed samples. Similar to our findings, Chon et al. (2018) detected higher *Campylobacter* prevalence rates in poultry meat samples by an enumerative assay than

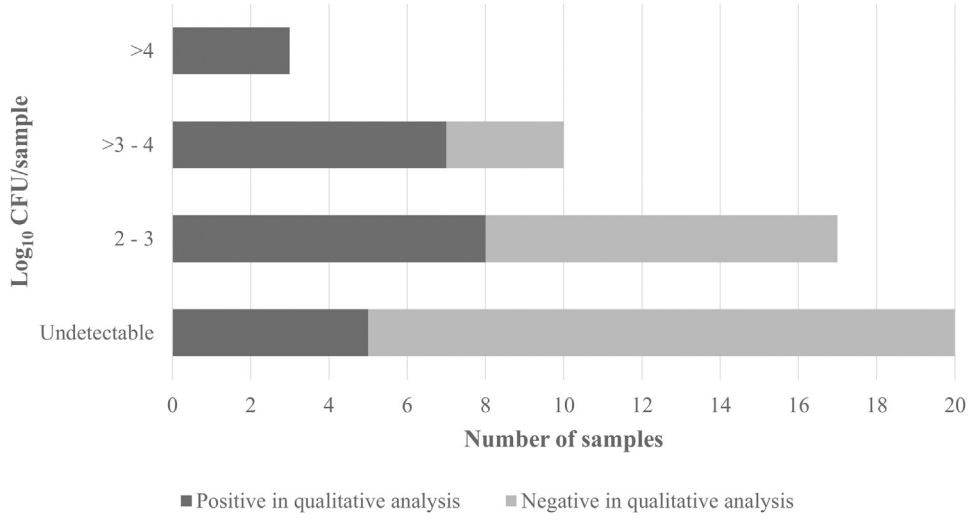

Figure 1. Distribution of thermotolerant *Campylobacter* levels in the chicken samples analyzed.

Table 1. Result of thermotolerant *Campylobacter* qualitative analysis in chicken samples based on the isolation strategy and isolated species.

Enrichment time	Selective plate medium	No. of positive/total samples[1]	Isolated species (no.)[1]
24 h	Preston agar	23/50[a]	*C. jejuni* (18)[a] *C. coli* (4)[b] *Campylobacter* spp. (1)
	mCCD agar	1/50[b]	*Campylobacter* spp. (1)
48 h	Preston agar	10/50[A]	*C. jejuni* (5)[A] *C. coli* (2)[A] *Campylobacter* spp. (3)
	mCCD agar	0/50[B]	-

Abbreviations: mCCD, modified charcoal–cefoperazone–deoxycholate.
[1]Values followed by different superscripts at each enrichment time (lowercase in 24 h and uppercase in 48 h) in different columns are significantly different ($P \leq 0.05$).

by a qualitative analysis with a selective enrichment step. As shown in Table 1, the alternative 24-h enrichment in Bolton broth before plating onto Preston agar was more effective at isolating *Campylobacter* from chicken compared with 48-h enrichment ($P = 0.0102$). Preston agar yielded more positive samples than mCCD agar after enrichment for 24 (23/50, 46%, $P < 0.0001$) and 48 h (10/50, 20%, $P = 0.0012$). Only one chicken sample was concurrently detected as *Campylobacter*-positive in mCCD agar and Preston

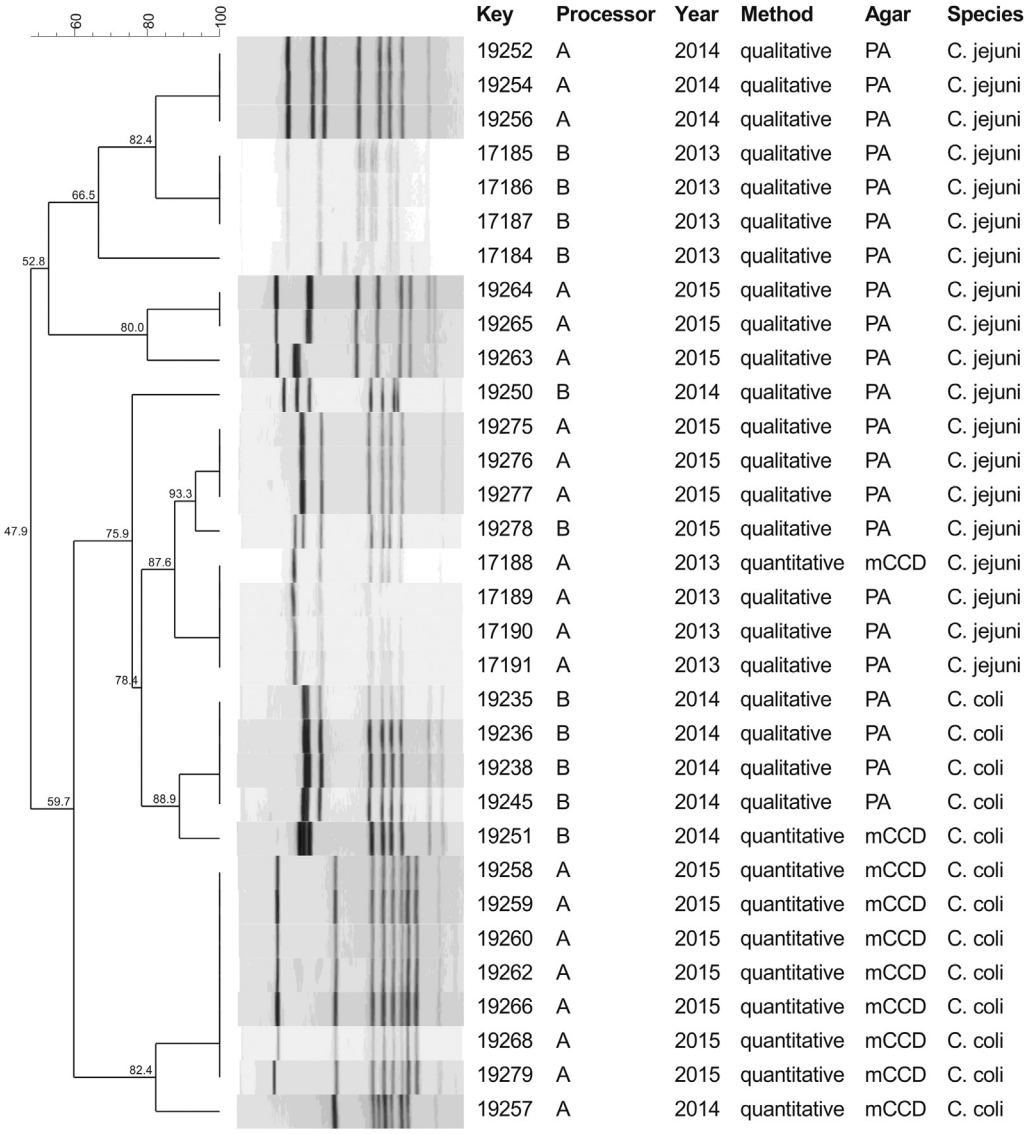

Figure 2. Cluster analysis of the pulsed-field gel electrophoresis subtypes found in the analyzed *Campylobacter* strains detected in retail samples sourced from different processors (A and B). Key: a randomly selected strain from the qualitative or quantitative analysis in individual chicken samples; PA: Preston agar; mCCD: modified charcoal–cefoperazone–deoxycholate agar.

agar (data not shown). Among the most common selective plate media to isolate *Campylobacter* from foods, mCCD agar is the best choice for analysis of chicken samples, offering an accurate and simple approach to identifying *Campylobacter* spp. (Gharst et al., 2013). Therefore, a higher selectivity efficiency of mCCD agar than that observed would have been expected in this study.

Enriched samples plated onto mCCD agar showed extensive spreading of competing bacteria, which hampered the identification of *Campylobacter* colonies in the qualitative analysis, especially after 48-h enrichment. All the analyzed contaminant strains recovered from mCCD agar were phenotypically characterized as nonproducing ESBLs *Proteus mirabilis*. Multidrug-resistant *P. mirabilis* showing β-lactamases genes have been previously isolated from chicken carcasses (Sanches et al., 2019) and reported on *Campylobacter* cultures by next-generation DNA sequencing analysis (Oakley et al., 2012).

The isolated *P. mirabilis* strains showed MICs of ≥62.5, ≥125, and ≥1,000 μg/mL to polymyxin B, cefoperazone, and trimethoprim, respectively, which were above the concentrations present in the Bolton broth and selective agar used. However, the MIC found for rifampicin (≤5 μg/mL) was below the concentration used in Preston agar (10 μg/mL). The resistance to cefoperazone would explain the spread of *P. mirabilis* on mCCD agar after enrichment in Bolton broth and the higher efficacy of Preston agar to inhibit contaminant strains sensitive to rifampicin. Owing to the high level of contaminants resistant to third-generation β-lactams used in *Campylobacter*-selective broth and agar culture media, the addition of rifampicin (Chon et al., 2017), potassium clavulanate (Moran et al., 2011; Seliwiorstow et al., 2016), or triclosan (Seliwiorstow et al., 2016) to Bolton broth was previously proposed to improve *Campylobacter* detection in chicken samples. Moreover, it has led to the recent revision of the ISO method for the analysis of samples with low *Campylobacter* numbers and a high level of background microflora, by introducing the enrichment of the test portions in Preston broth for 22 to 26 h (Biesta-Peters et al., 2019).

In total, 34 thermotolerant *Campylobacter* isolates were obtained from enriched samples, with a higher frequency of *C. jejuni* than *C. coli* ($P = 0.0028$, Table 1) detected in Preston agar after 24-h enrichment. A limited number of isolates were lost before the complete phenotypic characterization and thereby identified as *Campylobacter* spp. (Table 1). Additional assays, such as PCR, improve the discrimination between *Campylobacter* strains (Gharst et al., 2013). However, consistent with these findings, earlier research found that *C. jejuni* was more prevalent than *C. coli* in poultry samples (Giombelli and Gloria, 2014; Panzenhagen et al., 2016; Casagrande Proietti et al., 2018; Rossler et al., 2019). Note that in the present study, however, the majority of *C. jejuni* strains were isolated in Preston agar. The antimicrobial composition of selective media might select for particular *Campylobacter* species. As already reported, the polymyxin B present in Preston agar would impair *C. coli* isolation (Ng et al., 1985), which might explain the relatively higher frequency of *C. jejuni* in Preston agar. Such a finding reinforces the use of a second selective agar with different antimicrobial agents to avoid the *Campylobacter* population bias. Among the 32 strains subtyped, PFGE successfully discriminated between the *C. jejuni* and *C. coli* strains analyzed, with an evident difference between strains isolated in chicken sourced from both broiler processors sampled at retail, as the majority of strains could be associated with each sampling year (Figure 2).

In this study, *C. jejuni* and *C. coli* were identified in chicken at retail. The strains analyzed showed a distinct association with chicken sources. Although the combined frequency of *Campylobacter*-positive samples was 70%, the majority of samples showed <3 $\log_{10}$ CFU per portion. Good hygiene and thorough cooking of chicken can effectively prevent *Campylobacter* infection. Therefore, domestic food safety practices and consumer education continue to be required. Cefoperazone-resistant *P. mirabilis* strains impaired *Campylobacter* isolation from the samples enriched in Bolton broth before plating onto mCCD agar in the qualitative analysis. Nevertheless, the direct inoculation of the serially diluted test portions onto mCCD agar most probably avoided the contaminant background microbiota and resulted in the highest *Campylobacter* frequency in the quantitative analysis. The results underline the effect of the spread of contaminant strains onto *Campylobacter* cultures, which might be prevented using a recently revised ISO method for qualitative analysis of chicken.

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