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Quantitative and Qualitative Evaluation of *Campylobacter* spp. Contamination of Turkey Cecal Contents and Carcasses during and following the Slaughtering Process

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ABSTRACT

The present study aimed to document quantitatively and qualitatively the contamination by thermotolerant *Campylobacter* spp. of turkey samples during slaughtering. Four *Campylobacter*-positive turkey flocks were investigated at the slaughterhouse at three different stages: evisceration (cecal content), after carcass rinses but before chilling (neck skin), and after breast meat cut (meat). In each case, the studied flock was slaughtered first thing in the morning any given day of the week. The efficiency of cleaning and disinfecting operations was examined in the facility prior to processing the studied flock. For each flock, 90 samples were collected from cecal contents, neck skins, and meat pieces and checked quantitatively and qualitatively for *Campylobacter*. Identification of *Campylobacter* species was determined by PCR, and genetic patterns were determined by pulsed-field gel electrophoresis. *Campylobacter* contamination levels of ceca range from 2 to more than 7 Log CFU/g, while those of neck skin range from 0.5 to 3.5 Log CFU/g and those of meat range from 0.1 to 1.9 Log CFU/g. These differences in *Campylobacter* counts were not associated with a modification of *Campylobacter* species ratio; however, in the *Campylobacter jejuni* population, four genetic groups identified from the ceca were not recovered during slaughtering operations and two other genetic groups were only detected after chilling at the cutting stage of the breast meat. The present study suggests that the slaughtering process did not affect *Campylobacter* species populations; however, it might have influenced the strain population. Finally, the *Campylobacter* populations found on breast meat were similar to those isolated from the digestive tract of the birds.

Campylobacter spp. are the leading cause of human enteritidis infections worldwide *(20).* Campylobacteriosis has been associated with outbreaks *(14, 23, 24, 41, 42),* but it is more commonly associated with sporadic cases. Consumption and handling of poultry *(3, 35),* and more particularly the consumption of undercooked poultry meat or the occurrence of cross-contamination during poultry handling *(24),* have been identified as the most significant risk factors for *Campylobacter* transmission to humans.

Campylobacters have been identified in the gut of a wide range of animals *(29).* They are located mainly in their lower gastrointestinal tract, especially within the cecal content, where *Campylobacter* can reach a population density approaching 10^7 cells per g *(6, 26).*

Although the origins of *Campylobacter* contamination remain unclear at the farm level, its rapid proliferation in the flocks is thought to be due either to coprophagic behavior in poultry *(34)* or to the use of drinking nipples *(7).* Contamination and cross-contamination can also occur during feed withdrawal periods *(16),* the transportation of birds *(25, 50),* and all the associated stresses before slaughtering *(2, 36, 37).* It is widely acknowledged that any slaughtering event, and more particularly the defeathering and evisceration, can lead to the spreading of contamination onto carcasses *(21, 49)* and that flocks can cross-contaminate due to contacts with common surfaces within the environment *(33, 39).*

Little is known about the *Campylobacter* status of conventional turkey slaughterhouses in France. The objectives of the present study were to (i) assess the amounts of *Campylobacter* found on turkey carcasses during slaughtering and portioning in order to provide results that could be used for quantitative risk assessments and (ii) determine by pulsed-field gel electrophoresis (PFGE) the main genotypes present on the carcasses and track their introduction during slaughtering and further processing operations.

MATERIALS AND METHODS

Bacteriological sampling. Four commercial turkey flocks, originating from different farms in Brittany (France), were sampled in July (flock 1), September (flock 2), October (flock 3), and November (flock 4) during the year 2005 on four separate visits to two commercial abattoirs. In those abattoirs, carcasses were mechanically opened on a carousel: the cloaca was removed by the machine, and the carcass was opened with an automatic cutter. After that, the gut was removed manually. Each of the four flocks was slaughtered at the beginning of the daily slaughtering operations any day of the week, mainly to avoid cross-contamination. Each flock sampled consisted of about 2,900 to 5,000 birds.

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For each sampling session, environmental samples were taken before and during the processing of the flock. Ready-to-use surface sampling kits (Sodibox, La Forêt Fouesnant, France), consisting of a sterile water-moistened cloth, were used to wipe the intestine transport belt at the slaughterhouse and the cutting tables at the meat cutting plant. Aliquots (50 ml) of scalding water were also sampled (scalding water temperature ranged from 51 to 52°C) before and during the processing of the third flock (after processing ca. 1,000 birds).

For each of the four flocks, samples consisted of 30 ceca removed postevisceration, 30 neck skins sampled from carcasses just before chilling, and 30 turkey skinless, boneless breast meat samples harvested after chilling (approximately overnight) in the meat cutting part of the plants.

All samples were individually put into stomacher bags and stored at approximately 4°C during sampling and transportation to the laboratory without using transportation medium as the time between the end of sampling operations and arrival to the laboratory was less than 2 h. All the samples were treated at the laboratory on the day of sampling.

Growth conditions and media. All *Campylobacter* strains were cultured routinely at 41.5°C in a microaerobic atmosphere (7% O_2, 10% CO_2, and 83% N_2). Environmental swabs were supplemented with 100 ml of antibiotic-free Preston broth *(12)* (Oxoid, Dardilly, France) containing 10% of a neutralizing solution (Lab. Humeau, La Chapelle-sur-Erdre, France) and incubated for 4 h under microaerobic conditions at 37°C. Then, Preston antibiotics (AES Laboratory, Combourg, France) were added to the samples and incubated at 41.5°C under microaerobic conditions.

Ten grams of ceca (cecal membrane and cecal content) or neck skins and 25 g of turkey breast meats were added to 90 and 225 ml of Preston broth (Oxoid), respectively. All the samples were homogenized by stomaching 2 min (AES Laboratory). Samples were cultured either following enrichment (qualitative approach) or by direct plating (quantitative approach). For enrichment, cecum, neck skin, or turkey breast suspensions in Preston broth were incubated for 18 to 24 h at 41.5°C under microaerobic atmosphere (7% O_2, 10% CO_2, and 83% N_2). Following this enrichment step, a 10-μl sample of each Preston broth was streaked onto a Virion medium (Mueller-Hinton agar) (Merck Coger, Paris, France) with Bacto agar (Difco, Fisher Scientific, Elancourt, France) and 5% defibrinated horse blood (AES Laboratory) *(22)* and onto a Karmali medium *(27)* and incubated for 48 h under the same gaseous atmosphere. For the quantitative approach, serial dilutions of each sample (Preston broth) were performed in Trypton Salt Broth MRD-T (Biomérieux SA). Subsequently, 50 μl of the appropriate dilutions were automatically inoculated onto Karmali plates by using spiral plating according to the WASP method (Don Witley Scientific Limited). Colony counting was performed after 72 h of incubation at 41.5°C under microaerobic atmosphere (7% O_2, 10% CO_2, and 83% N_2). One randomly selected colony from each positive sample was stored in Brucella broth (Difco) supplemented with 15% (vol/vol) glycerol at −70°C for subsequent analysis.

Statistical analysis. Significance of the differences in percentage of contamination by *Campylobacter* between batches at the same stage was tested by the χ^2 test.

Molecular subtyping by multiplex PCR and PFGE. At least one colony was selected per positive sample. Considering that some studies have highlighted that subtype distribution is affected by direct plating or enrichment, we favored colonies obtained from direct isolates. Indeed, the enrichment step may provide an opportunity for rapidly growing strains to outgrow slow-growing ones *(2, 19, 33)*.

A bacterial subculture of each colony was undertaken, and several colonies from the bacterial culture were suspended in 200 μl of Tris-EDTA (TE) buffer (10 mmol $liter^{-1}$ Tris-HCl, 1 mmol $liter^{-1}$ EDTA, pH 7.6) and used for PCR. The remaining colonies were used for genotyping by restriction fragment length polymorphism–PFGE, as described hereafter.

Simultaneous PCR identification of *Campylobacter jejuni* and *Campylobacter coli* was performed as described by Denis et al. *(18)* using a Gene AMP system 9600 (Perkin Elmer Instruments, Norwalk, CT). In order to obtain visualization of PCR products, a 10-μl aliquot of each amplification product was electrophoresed in a 1.5% agarose (Eurobio, Les Ulis, France) gel stained with ethidium bromide for 2 h at 100 V. The images were captured under UV illumination by a video system (gel DOC 1000 system, Biorad).

The typing of each strain was realized by macrorestriction analysis using PFGE as described by Rivoal et al. *(39)*. Bacteria were suspended in 2.5 ml of TN (Tris HCl, pH 7.5, 10 mM containing 1 M NaCl). Cells were washed twice with TN and resuspended in an adequate volume of TN in order to obtain an optical density at 600 μm of 1.5. Agarose plugs were prepared by adding to 180 μl of cell suspension an equal volume of 1.5% low-melting-point fusion agarose (LMP agarose, Eurobio) prepared with distilled water. The agarose-cell mixture was poured into plug molds. The resulting plugs were incubated from 40 h to fewer than 72 h in a lysis buffer. After the lysis step, plugs were washed with a TE buffer and sliced into four equal pieces. A quarter plug was used to restrict endonuclease digestions in separate reactions using 40 U of either *Sma*I or *Kpn*I (Boehringer) under the manufacturer's conditions, in a final volume of 100 μl for 5 h of incubation at the appropriate temperature. PFGEs were conducted using the CHEF-DRIII system (Biorad Laboratories, Hercules, CA). The agarose gel (1%) prepared in 0.5× TBE (Tris, 45 mmol $liter^{-1}$; boric acid, 45 mmol $liter^{-1}$; EDTA, 1 mmol $liter^{-1}$) was subjected to electrophoresis (23 h at 220 V, 14°C, with ramped pulse times from 2 s to 25 s for *Kpn*I; 24 h at 200 V and 14°C, with ramped pulse times from 15 to 45 s for the first 22 h and from 2 to 8 s for the last 2 h for *Sma*I).

The agarose gels were stained with ethidium bromide, and the images were captured under UV illumination by a video system (gel DOC 1000 system, Biorad).

Electrophoretic patterns were compared by BioNumerics (Applied Maths). Similarities between profiles, based on band positions, were derived from the Dice correlation coefficient with a maximum position tolerance of 1%. A dendrogram of the analysis of the combined *Kpn*1- and *Sma*1-digested DNA was constructed to reflect the similarities between the strains in the matrix. Strains were clustered by the unweighted pair group method using the arithmetic mean *(46)*.

In this study, clusters were defined as having a genetic similarity equal or superior to 80% *(39)*.

Isolates with genetic similarity under 50% were considered different. Isolates with high similarity were considered as deriving from the same parent strain.

RESULTS

Qualitative and quantitative analysis of the contamination. Among the three kinds of samples (i.e., cecum, neck skin, and turkey breast meat), ceca were the most

TABLE 1. *Percent positive samples per sample type harvested from flocks 1 through 4 and number of* Campylobacter *organisms*[a]

Flock no. (abattoir, mo)	% of positive samples (no. of *Campylobacter* organisms [mean Log CFU/g])		
	Ceca	Neck skin	Breast meat
1 (A, July)	100.0 (6)	96.7 (1.1)	83.3 (0.9)[b]
2 (B, September)	73.3 (2.1)[b]	90.0 (1.0)	46.7 (0.5)[b]
3 (A, October)	100.0 (7.2)	100.0 (3.5)	100.0 (1.9)
4 (B, November)	100.0 (6)	43.3 (0.5)[a]	3.3 (0.1)[b]

[a] For breast meat, flock 4 < flock 2 < flock 1 < flock 3 (by the χ^2 test).
[b] Flock is significantly less contaminated at 1% (by the χ^2 test).

TABLE 2. *Number of positive samples obtained during 24 h in Preston broth*[a]

Flock no.	No. of positive samples								
	Ceca			Neck skin			Meat		
	I	II	T	I	II	T	I	II	T
1	30	0	30	28	1	29	0	25	25
2	20	2	22	6	21	27	3	11	14
3	30	0	30	30	0	30	28	2	30
4	29	1	30	5	8	13	1	0	1

[a] I, before enrichment; II, after enrichment; T, total number of positive samples.

contaminated (Table 1). Indeed, *Campylobacter* was detected in at least 73% of the ceca by direct plating from the Preston broth suspension, while detection was poor when using enrichment prior to isolation (Table 2). *Campylobacter* was found in 90 to 100% of the sampled neck skins issued from flocks 1, 2, and 3. Breast meat samples were also contaminated, but an increased number of samples required a 24-h enrichment period in Preston broth, as shown in Table 2, to be detected (38 showed contamination after enrichment versus 32 by direct plating).

To quantify the contamination, CFU were recorded from each sample. In both slaughterhouses there was a significant difference between the contamination within the ceca, which could reach 5×10^8 CFU/g of cecum, and the resulting contamination present on the breast meat (Table 1), which remained below 10^2 CFU/g. Flock 2 exhibited a lower density of *Campylobacter* in the ceca. Only 5 samples of the 30 tested resulted in more than 10^3 CFU/g of cecum, and those remained under the level of 10^7 CFU/g. Neck skin samples from flock 2 were colonized at less than 10^2 CFU/g. Interestingly, this flock exhibited only 46.7% of contaminated breast meat carrying low amounts of bacteria (less than 120 CFU/g). Flock 4 exhibited only 43.3% of contaminated neck skins, and the quantified levels of bacteria were less than 250 CFU/g of neck skin, while *Campylobacter* spp. were detected on 3.3% of the breast meat samples.

Environment sampling before processing. The persistence of campylobacters in the processing environment was tested before the start of the processing. All sampled surfaces and knifes tested negative for *Campylobacter*. During the processing, contamination was detected on cutting boards and knives (Fig. 1). Scalding water (51 to 52°C) was tested for contamination by *Campylobacter* prior to and during the slaughter of flock 3. Before any turkey was processed, no *Campylobacter* could be detected. However, campylobacters were detected as soon as 1,000 turkeys were processed through the scalding tank.

Subtyping of the collected strains. *C. jejuni* was the predominant species encountered in flocks 1, 2, and 3, while *C. coli* was dominant in flock 4. Indeed, in flocks 1 and 2, 100% of the collected strains from ceca, neck skins, and breast meat belonged to the *C. jejuni* species. Both *C. jejuni* and *C. coli* were isolated from flock 3, and *C. coli* represented only 18.2% of the isolates from ceca (Table 3). Overall 83% of isolates from flock 4 were *C. coli*.

Genotype of the collected isolates. Among the 168 PCR subtyped isolates collected in this study, 154 were genotyped. Except within flock 1, a limited number of PFGE types were isolated from the different flocks, but PFGE types were different between flocks. Eleven *Sma*I-*Kpn*I PFGE types were identified from flock 1 and can be clustered within five distinct groups. The *C. jejuni* (J) isolates of type J20, J19, J18, or J17 share more than 91% similarity. They were obtained from ceca and neck skin but were never recovered from the skinless breast meat. This cluster is distantly related to the other groups and shares less than 37% similarity with the other groups. Surprisingly, some strains, such as J6 or J5, were not recovered from ceca or neck skin but were obtained after chilling at the cutting stage of the breast meat (Fig. 1). Only one isolate was genotyped from flock 2, leading to the identification of type J10. Most isolates collected from flock 2 had lost, for unidentified reasons, their ability to grow, which precluded PFGE. Flock 3 yielded two *C. coli* and five *C. jejuni* types. Type J13 represented 55, 96, and 83% of the genotyped isolates identified in ceca, neck skin, and breast meat, respectively. The *C. coli* (C) types C1 and C2 were detected in equal amounts in the isolates. As mentioned above, flock 4 was characterized by a contamination mainly due to *C. coli*. Indeed, type C3 was observed in more than 90, 60, and 100% of the ceca, neck skin, and breast meat of flock 4, respectively. Surprisingly, type C3 was the only *C. coli* type observed in this flock. *C. jejuni* strains isolated from flock 4 yielded a cluster containing three types (Fig. 1). Types J7, J8, and J9 are closely related and share 92% similarity.

DISCUSSION

Most of the cecal samples positive for *Campylobacter* were recovered by direct plating rather than by enrichment procedures. This phenomenon has been described *(31)* and is probably due to the competing flora of the gut content overgrowing *Campylobacter* in enrichment broth.

In this study, no *Campylobacter* was recovered in the plant environment at any of the sampling dates before the

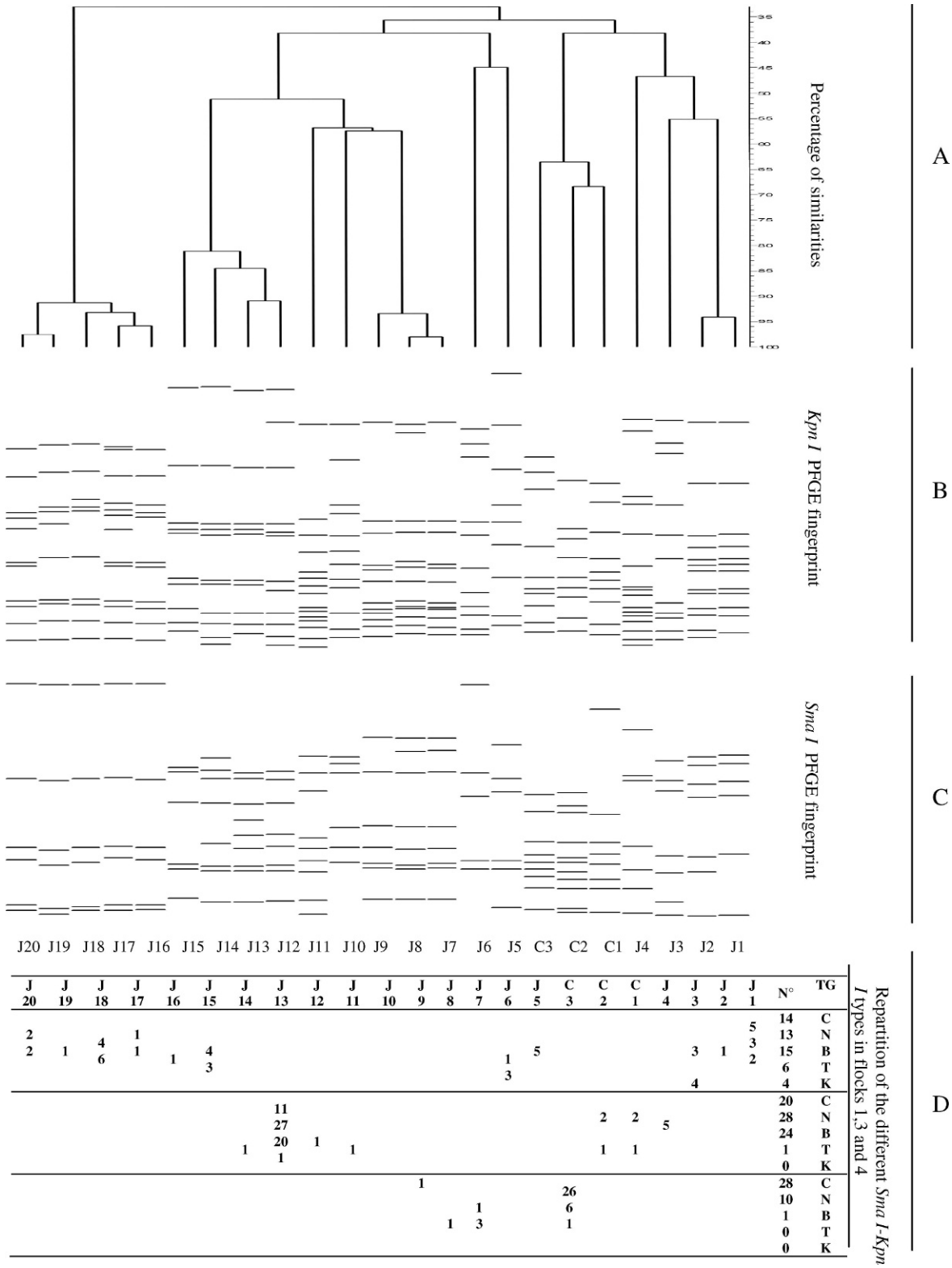

FIGURE 1. *(A) Clustering of composite data obtained from* Sma*I and* Kpn*I PFGE fingerprints. (B)* Kpn*I PFGE fingerprint. (C) Distribution of the different PFGE types in flocks 1, 3, and 4. The text columns show the flock numbers, the PFGE types, and the counts of the different genotypes per type of sample, i.e., ceca (C), neck skins (N), skinless breast meat (B), table swabs (T), and knives (K). The rightmost column exhibits the total number of genotyped strains per sample type (TG). Profile J10 was obtained in flock 2, which is not presented in this figure.*

TABLE 3. *Percentage of* C. jejuni *and* C. coli *for each flock and each sample type*

	% of *C. jejuni* or *C. coli* strains and no. of strains								
	Ceca			Neck skin			Breast meat		
Flock no.	*C. jejuni*	*C. coli*	*n*	*C. jejuni*	*C. coli*	*n*	*C. jejuni*	*C. coli*	*n*
1	100	0	13	100	0	15	100	0	15
2	100	0	9	100	0	9	100	0	11
3	81.8	18.2	22	100	0	23	90	10	10
4	13.8	86.2	29	36.4	63.6	11	0	100	1

beginning of slaughtering operations, suggesting that daily cleaning and disinfection procedures are effective and efficient. These results are corroborated by previously published data *(17).* It is widely accepted that cells adapt to survive in such harsh environments. *Campylobacter* has previously been described to have the ability to adopt a viable but nonculturable physiological state *(5, 40, 43).* We cannot exclude the presence of viable but nonculturable forms of *Campylobacter* in this environment, which would not be detected under the protocols that we applied.

During the third sampling experiment, scalding water (ca. 51 to 52°C) was analyzed before and during the processing of flock 3. Before the start of the process, no *Campylobacter* was detected. After at least 1,000 birds had been processed, the scalding water was found to be contaminated with *Campylobacter* type J13, the most frequently occurring strain in this flock. The survival of *Campylobacter* within scalding water had previously been observed *(7, 30, 45).* In a study performed by Alter et al. *(2),* it was shown that noninfected flocks could become contaminated on the surface by the scalding water bath used for previously slaughtered flocks. This phenomenon, combined with feather and skin contamination prior to slaughter *(9),* contributes to the spread of contamination onto carcasses prior to evisceration *(39),* even if scalding reduces the total number of bacteria on the skin, including *Campylobacter (10).* Therefore, logistic slaughtering could present a good choice to prevent cross-contamination.

Some studies have recently highlighted the need to better understand the quantitative distribution of *Campylobacter* contamination during processing *(4, 28).* These articles focused on carcass or meat contamination but lacked quantitative information on the status of the birds or primary production. Many studies have described a high level of contamination at the farm level *(47).* Some of those studies underlined the role of the crop contamination as a source of *Campylobacter* entering the processing plant *(15)* and of feed withdrawal responsible for the increase of *Campylobacter* contamination of the crop *(16).* This increase of *Campylobacter* contamination of the crop due to feed withdrawal may be associated with a decline in lactic acid in the emptied crop *(51).* The distribution of contamination from flock to carcass and to meat is mainly regarded with a qualitative approach *(13, 26).* Some studies investigated the number of *Campylobacter* in broiler cecal contents *(9)* and found contamination levels close to those we obtained on turkeys. Recent investigations on the subject have emphasized the need for such data from a quantitative risk assessment perspective *(32).* Our work originally described the *Campylobacter* quantitative contamination of cecal content, skin, and meat from identified and individually monitored turkey flocks. Nevertheless, the slaughtering and cutting operations led to low amounts of *Campylobacter* on the final skinless breast meat. These results suggest that the slaughtering process could be efficient in limiting the spread of the contamination. This suggests that even in the most favorable situations, the slaughtering process cannot avoid the spread of contamination but such spread can be controlled by implementing good manufacturing practices. Bhaduri and Cottrell *(11)* have highlighted that refrigeration and frozen storage would not add significantly to the safety advantages and cannot replace sanitary production and handling. Nevertheless, Berrang et al. *(8)* have demonstrated that spin chilling of carcasses is associated with a 2-log decrease of *Campylobacter* contamination.

The determination of the *Campylobacter* species involved in the contamination has highlighted differences in the four flocks. In contrast to flock 4, where *C. coli* predominated, all the others were contaminated mainly by *C. jejuni*. When *C. jejuni* was found to be predominant in the ceca, it remained the most frequently encountered contamination on the neck skins and breast meat. Similar observations were made for *C. coli* (flock 4). The differences between species levels of contamination of a tested flock are thought to occur during the rearing period and are illustrated by the diversity observed within the cecal contents.

PFGE has shown that the encountered genotypes vary drastically between flocks *(48).* Strain diversity can lead to as little as 35% similarity between clusters. One flock (the fourth) was mainly contaminated with one *C. coli Sma*I-*Kpn*I PFGE fingerprint. Both *C. jejuni* and *C. coli* were identified from flock 3, but type J13 was the most frequently encountered contamination. Within flock 1, which exhibited at least 11 distinct profiles of *C. jejuni* and showed no *C. coli,* strains that belonged to types J20, J19, J18, or J17 represented more than 50% of the isolates from ceca or neck skins. Nevertheless, these types were not identified either on breast meat samples or on environmental swabs. These four types belonged to a cluster sharing less than 37% similarity with the others. Clonal groups sharing specific characteristics with regard to their ability to colonize or to survive stresses have previously been described *(33).* Further physiological experiments will be required to analyze the

adaptability characteristics of such isolates when facing environmental stresses. Thus, slaughtering process may not have a drastic effect on species selection but may have more of an effect at the strain level.

Some types do appear at different stages of the process. Indeed, types J5 and J6 were not detected within ceca or neck skin but represented 40% of the isolates identified in breast meat.

The occurrence of new types of strains during processing, as suggested by Rivoal et al. *(39),* should also be due to cross-contamination. Despite the facts that the environmental swabs did not reveal any contamination and that each of the four flocks studied was the first slaughter of the day, we cannot exclude that live animals hosted more strains than we identified, that our sampling from the slaughtering process was not exhaustive, or that aerosols were responsible for the contamination of carcasses. Furthermore, a recent study highlights that no cross-contamination is observed for the flock that is processed first on any given day *(1).*

The disappearance of the main contamination, as illustrated by the cluster J17-18-19-20 in flock 1, could promote the selection and detection of preexisting but common lesser genotypes. This hypothesis is strongly supported by previously published works that highlight how varying abilities to survive environmental stresses can result in differing capabilities for colonization and the spread of contamination *(38, 44)*. These new isolates from a new type should also have been selected by the enrichment medium: within flock 1 isolates from ceca and skin resulted from direct plating, while isolates from meat were issued from enrichment procedure (Table 2).

As it has often been suggested, *Campylobacter* contamination of poultry carcasses mainly originated from live birds that were contaminated during the rearing period. Reducing the amount of *Campylobacter* in the gut at the end of the rearing period may reduce the risk of heavily contaminated carcasses.

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