Campylobacter jejuni *and* Campylobacter coli: *prevalence, contamination levels, genetic diversity and antibiotic resistance in Italy*

Simona Iannetti*, Paolo Calistri, Gabriella Di Serafino, Francesca Marotta, Alessandra Alessiani, Salvatore Antoci, Diana Neri, Margherita Perilli, Giorgio Iannitto, Luigi Iannetti, Giacomo Migliorati and Elisabetta Di Giannatale

Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise 'G. Caporale', Campo Boario, 64100 Teramo, Italy

**Corresponding author at: Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise 'G. Caporale', Campo Boario, 64100 Teramo, Italy.*
E-mail: s.iannetti@izs.it

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Broiler carcasses, *Campylobacter*, Contamination levels, Production chain, PFGE.

Summary
A research was carried out in Italy with the aim of assessing *Campylobacter* contamination in broilers from breeding to slaughter, of defining the genetic diversity of isolates and their antibiotic resistance. Sampling was carried out in a slaughterhouse, and in farms representative of the most common broiler production in Italy. At farm, the 78.8% (95% C.I.: 74.5%-82.5%) of cloacal samples tested positive for *Campylobacter* spp. *C. jejuni* showed higher prevalence in winter than in spring and summer ($p < 0.00001$, $\chi^2 = 32.9$), while *C. coli* showed an opposite trend ($p < 0.00001$, $\chi^2 = 41.1$). At slaughterhouse, the 32.3% (95% C.I.: 30.2%-35.2%) and the 23.9% (95% C.I.: 21.7%-26.3%) of skin samples tested positive for *C. jejuni* for *C. coli*, respectively. *C. coli* showed higher prevalence than *C. jejuni* at washing ($p < 0.05$, $\chi^2 = 11.11$) and at chilling ($p < 0.05$, $\chi^2 = 9.26$). PFGE revealed high heterogeneity among isolates. Some clones were identified within the same farm in more than one season, suggesting environmental conditions able to support their persistence; other clones resulted to be spatially distant, suggestive of cross-contamination. Both *Campylobacter* species showed high resistance to nalidixic acid and ciprofloxacin, while resistance to erythromycin was more frequent in *C. coli* than *C. jejuni* ($p < 0.05$; χ^2 test).

Introduction

Campylobacter jejuni and *coli* are the most important cause of bacterial gastroenteritis worldwide infecting humans mostly through consumption of contaminated poultry (Dingle *et al.* 2002, Food *et al.* 2014). *Campylobacter* species colonise the gastrointestinal tract of domestic and wild animals and their prevalence in food producing animals, such as cattle, swine and poultry, can exceed the 80% (Mughini Gras *et al.* 2012, EFSA 2017). *Campylobacter* has a broad host range and has been detected everywhere, from farm and urban environments to slaughter plants, in wild birds and mammals, companion animals and farm production animals (Whiley *et al.* 2013, Alter *et al.* 2005, Pearce *et al.* 2003). *Campylobacter* species are highly adapted to asymptomatically colonise the intestinal tract of most avian species, reaching high numbers (up to 10^{10} cfu/g caeca content) in chickens and turkeys (Newell *et al.* 2008). Once *Campylobacter* is introduced into a flock, it spreads quickly. Indeed, it can reach a within-flock prevalence ranging from 60% to 100% (Barrios *et al.* 2006). Higher prevalence of infection has been observed in many countries in warmer months, suggesting a seasonal pattern in the colonization of poultry flocks (Horroks *et al.* 2009). The reason behind this seasonal effect is largely unknown, although a possible role of migratory birds or insects has been suggested (Jacobs-Reitsma 1997). *Campylobacter* infections in colonized flocks could be transmitted horizontally within the farm via a variety of routes and vehicles. The possible primary infection sources and transmission routes of *Campylobacter* for poultry flocks have been investigated in numerous studies, but no definitive factors have been identified so far that explain the high levels of prevalence observed in commercial poultry flocks. Risk factors associated with the introduction and dissemination of *Campylobacter* within the flocks may include lack of biosecurity

measures, contaminated water or feed, contacts with other infected animal species (wild birds, pets, mice, etc.) and mechanical transmission via insects (Barrios *et al.* 2006, Horroks *et al.* 2009). Some authors have suggested that *Campylobacter* can spread from the parent flocks to the progeny (Cox *et al.* 2002, Petersen *et al.* 2001b, Sahin *et al.* 2003, Shanker *et al.* 1986) although some observations indicate that vertical transmission plays a minor role in *Campylobacter* flock colonization (Petersen *et al.* 2001a, Callicott *et al.* 2006). In the European Union (EU), enteric infections caused by *Campylobacter* are the most frequently reported zoonosis in humans; in 2016, 246,307 cases of campylobacteriosis have been reported in the EU, with an increase of 6.1% compared with 2015. (EFSA/ECDC 2017). The majority of *Campylobacter* infections in humans originate from the consumption and handling of raw or undercooked poultry meat products. In Italy, a recent study aimed at investigating an outbreak of campylobacteriosis, showed that in the 70% of cases of *C. jejuni* infection, the MLST profiles associated with human disease were most similar to those associated with chicken source (Di Giannatale *et al.* 2016). This result is in line with many other European countries, even if the percentage of human campylobacteriosis due to the chicken source vary among the studies (Wilson *et al.* 2008, Mullner *et al.* 2009, Sheppard *et al.* 2009). Therefore, the control of *Campylobacter* in poultry flocks and the reduction of poultry meat contamination are the cornerstones for any public health strategy aiming at reducing the incidence of campylobacteriosis in humans (EFSA 2008). In the EU, during 2008 a harmonised and standardised baseline survey on the prevalence of *Campylobacter* in broiler flocks and broiler carcasses was carried out, which assessed a prevalence of *Campylobacter* contamination in broiler carcasses in Italy equal to 49.6% (95% C.I. 39.5%-59.7%) (EFSA 2010b). The results of this survey showed that a *Campylobacter*-colonised broiler batch was about 30 times more likely to have the sampled carcass contaminated with *Campylobacter*, compared to a non-colonised batch, and that the risk of carcasses contamination increases from July to September (EFSA 2010c). According to relevant studies, faecal contamination of carcasses during slaughtering represents the main source of *Campylobacter* in fresh poultry meat (Mahler *et al.* 2011, Guerin *et al.* 2011). However, the main factors responsible for *Campylobacter* presence on carcasses have not been identified yet. Moreover, previous studies comparing *C. jejuni* and *C. coli* concentrations at farm and along the slaughtering chain are few (Schets FM *et al.* 2017). Given the results of the baseline survey, the European Food Safety Authority (EFSA) recommended the EU Member States to identify more clearly the risk factors of carcasses contamination during slaughtering and *Campylobacter* colonisation at primary production level (EFSA 2010c).

The aim of this research was to study the prevalence of *C. jejuni* and *C. coli* infection in chickens at farm and the contamination levels of carcasses in a typical Italian chicken production and slaughtering chain. Moreover, this study aimed at evaluating the antibiotic susceptibility profiles, the survival and genetic diversity of *Campylobacter* isolated at farm and at different stages of the slaughtering process using PFGE.

Materials and methods

Sample collection

The sampling activities were carried out in one slaughterhouse (slaughter capacity about 110,000 chickens per day) and in three farms located in Abruzzi region (Central Italy). The farms (A, B, C) selected belonged to the same company and were representative of the most common intensive broiler farms in Italy, on the basis of the breeding management system, the animal housing system, feeding programs and sanitary protocols. In such farms, broilers are usually grown as mixed-sex flocks in large sheds under intensive conditions; they are reared on ground on deep litter consisting in chopped straw and fed ad libitum with different feed formulas, depending on different stages of the animals' growth. The temperatures (24-33 °C), relative humidity (80-100%) with natural daylight of 12 h and artificial lighting during 12 h of darkness, are differently settled in relation of animals' age.

Sampling was carried out twice for each season (with the exception of August and March for logistic reasons) to take into consideration possible seasonal variations in the infection rates:

- Winter: from December to February;
- Spring: from April to May;
- Summer: from June to July;
- Autumn: from September to November.

At farm, sampling activities were performed during four breeding cycles in farms A, B and C. In particular, samples were collected at different stages of the breeding cycle:

- fifteen days before day-old chicks restocking (water trough, feed trough, fan blades, ground and insects of the species *Alphitobius diaperinus*, litter beetles commonly found in poultry houses);
- during day-old chicks restocking (shipping containers swabs, starter feed, manure);

- thirty days after restocking (water trough, feed trough, fan blades, growth feed and the water from the lake located inside the farm and used for chickens).

One day before slaughtering, fifty broilers for each batch were identified before leaving the farm with numbered leg-rings, and cloacal swabs were collected from each identified animal: the number of broilers identified was increased to take into account the possible lack of the leg-ring during the slaughtering operations. The identification of the broilers allowed preserving the link between the animal sampled before and during the slaughtering process.

At slaughterhouse, samples were collected from the neck skin of each identified carcass by excision after bleeding, defeathering, evisceration, washing, chilling. Caeca samples were also collected from the same carcasses after the evisceration stage.

Culture conditions and PCR assays

Campylobacter strains were recovered from skin after the enrichment according to ISO 10272-1:2006 and to ISO 10272-2:2006 methods. Caeca contents were directly plated on mCCD agar and incubated at 42 °C for 48 h under micro-aerobic conditions. For enumeration, 1 ml of caeca contents was added to 9 ml of peptone water pH 7.0 (1:10 g/ml), log-dilution were performed (until 10^{-9} dilution), and the plates were incubated at same conditions. The suspected colonies were then examined according to ISO 10272 method and confirmed by multiplex PCR (Wang *et al.* 2002). *Campylobacter* isolates were cultured on Columbia blood agar in microaerophilic conditions at 42 °C for 48 hours. Species identification was performed using a multiplex PCR (Wang *et al.* 2002). DNA from *Campylobacter* strains was extracted using the Maxwell 16 tissue DNA purification kit (Promega Corporation, Madison, WI) according to the manufacturer's instructions. All isolates were stored at - 80 °C.

One thousand ml of water lake was filtered using membrane filter (pore size 0.45 micron, Millipore). After filtration, membrane filter was placed in Bolton enrichment broth for 48 h at 42 °C in microaerobic condition, later streaked on mCCD agar plate for isolation, and then incubated again for 48 h at 42 °C in microaerobic condition. The suspected colonies were identified by multiplex PCR (Wang *et al.* 2002).

Antimicrobial susceptibility

The susceptibility of *Campylobacter* isolates to seven antimicrobials was evaluated with a micro broth dilution method using the 'Sensititre' automated system (TREK Diagnostic Systems, Biomedical Service, Italy). Colonies were cultured on Columbia agar for 48 hours in micro-aerobic atmosphere, inoculated in Mueller Hinton Broth supplemented with blood, and dispensed into Eucamp microtiter plates (TREK Diagnostic Systems, Biomedical Service, Italy). The plates contained known scalar concentrations of the following antimicrobial substances: gentamicin (Gm) (0.12-16 µg/ml), streptomycin (S) (1-16 µg/ml), ciprofloxacin (Cip) (0.06-4 µg/ml), tetracycline (Te) (0.25-16 µg/ml), erythromycin (ERY) (0-5-32 µg/ml), nalidixic acid (NA) (2-64 µg/ml), and chloramphenic (CPL) (2-32 µg/ml). The plates were then incubated at 42 °C in micro-aerobic atmosphere for 48 hours. *C. jejuni* strain NCTC 11351 was included for the quality control of the minimal inhibitory concentration (MIC) test. Antimicrobial resistance was interpreted according to CLSI breakpoints (CLSI Clinical and Laboratory Standards Institute).

PFGE

Pulsed-field gel electrophoresis (PFGE) was performed according to the instructions of the 2013 U.S. Pulse Net protocol for *Campylobacter* (Pulse Net International 2013). Strains of *C. jejuni* and *C. coli* were sub cultured on Columbia agar at 42 °C for 2 days in micro-aerobic atmosphere and embedded in agarose blocks (Seakem Gold agarose, Lonza, Rockland, USA). The blocks were then lysed, washed and digested with SmaI enzyme (Promega, Italy), 25 U at 25 °C for 4 hours. *Salmonella* serovar *Branderup* H9812 was used as standard molecular weight size. PFGE was performed using a Chef Mapper XA (Biorad Laboratories) with the following parameters: initial switch time of 6.75 s, final switch time of 35.38 s for 18 hours at 6 V and 14 °C in 0.5 X TBE buffer (Sigma). After electrophoresis, the gel was stained with Sybr Safe DNA gel stain (Invitrogen, USA) and photographed at transilluminator (Alpha Innotech, USA). Bionumerics v. 6.6 software (Applied Maths, Belgium) was used for the analysis of PFGE fingerprinting profiles. Level of similarity were calculated with the Dice correlation coefficient (position tolerance was set at 1%) and unweighted pair group mathematical average UPGMA clustering algorithm was used for cluster analysis of the PFGE pattern. PFGE-clusters were defined at 100% similarity between macro restriction patterns (Grotheus *et al.* 1991). Untypeable isolates were not included in the analysis.

Data collection and analysis

Sampling information was collected using specific sampling cards and recorded into Microsoft® Access database (MS-Access 2010) for further analyses. Microsoft® Excel (MS-Excel 2010) and XLStat©-Pro (Version 7.5) were used for descriptive and statistical analysis of the data.

Multiple Chi-Squared test was used to verify if in samples taken at slaughterhouse there were significant differences in prevalence levels among sampling seasons and sampling stages. Chi-Squared test was used to verify whether the prevalence levels observed for *C. jejuni* were significantly different from prevalence levels observed for *C. coli* in samples taken at slaughterhouse.

Contamination levels were checked for a normal distribution with the Kolmogorov-Smirnov test, and then non-parametric tests have been used because of non-normality of the data. Kruskal-Wallis test was used to verify significant differences among contamination levels of sampling seasons and significant differences among contamination levels of sampling stages in samples taken at slaughterhouse. Multiple comparisons among contamination levels of *C. coli* and of *C. jejuni* in skin and caeca samples were performed with Dunn Test.

Friedman test was used to verify if there were significant differences among contamination levels of skin samples taken from the same animal after bleeding, defeathering, evisceration, washing and chilling and multiple comparisons were performed with Nemenyi test.

Fisher F-test was also used to verify whether the contamination levels observed for *C. jejuni* were significantly different from contamination levels observed for *C. coli* in samples taken at slaughterhouse.

Results

Campylobacter prevalence and contamination levels

At farm, out of 400 cloacal samples, 315 tested positive for *Campylobacter* spp. (Prevalence = 78.8%, 95% C.I.: 74.5%-82.5%). *C. jejuni* showed higher prevalence levels than *C. coli* during each sampling season. A lower prevalence level was observed in spring than in the other seasons. *C. coli*, showed a different seasonal pattern with higher prevalence levels in spring than in the other seasons (Figure 1).

Multiple comparisons showed a significantly higher prevalence of contamination in winter than in spring and summer ($p < 0.00001$, $\chi^2 = 32.9$; Multiple chi-squared test) for *C. jejuni*, while *C. coli* showed a higher prevalence level in spring than in summer and autumn ($p < 0.00001$, $\chi^2 = 41.1$; Multiple Chi-squared test). The environmental samples, the feed and insects tested all negative for *Campylobacter* spp. by the detection method. The water collected from the lake located inside the farm 30 days after placement of the day-old chicks in the shed, and used for chickens, tested positive for *Campylobacter* spp. by filtration method and *Campylobacter coli* was identified by multiplex PCR. At slaughterhouse, 230 broilers out of 400 (57.5%) were sampled, for 1,333 samples (Table I). The rest of the broilers was not sampled due to the loss of the leg-ring during transport. The 32.3% (95% C.I.: 30.2%-35.2%) of skin samples tested positive for *C. jejuni* and the 23.9% (95% C.I.: 21.7%-26.3) of samples tested positive for *C. coli*. The 48.9% (95% C.I.: 42.9-55.8) of caeca samples tested positive for *C. jejuni* and the 28.9% (95% C.I.: 23.4-35.1) tested positive for *C. coli*. Figure 2 compares the prevalence of *C. coli* and *C. jejuni* contamination in samples taken at slaughterhouse. *C. jejuni* showed higher prevalence levels than *C. coli* in caeca samples, at bleeding, defeathering and evisceration, while after the evisceration stage

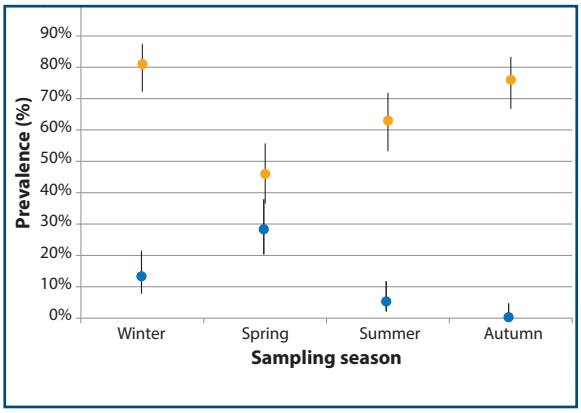

Figure 1. C. coli *(blue dots) and* C. jejuni *(orange dots): prevalence (± C.L. 95%) of contamination of samples taken at farm (cloacal swabs).*

Table I. *Number of samples taken at slaughterhouse.*

Month of sampling	Season	N. of animals	N. of caeca	N. of samples taken after the slaughtering stages					Total n. of samples
				Bleeding	Defeathering	Evisceration	Washing	Chilling	
Dec-Feb	Winter	46	46	46	46	46	46	45	275
Apr-May	Spring	60	55	55	56	55	59	55	335
Jun-July	Summer	68	68	68	63	63	63	62	387
Sep-Nov	Autumn	56	56	56	56	56	56	56	336
Total		**230**	**225**	**225**	**221**	**220**	**224**	**218**	**1,333**

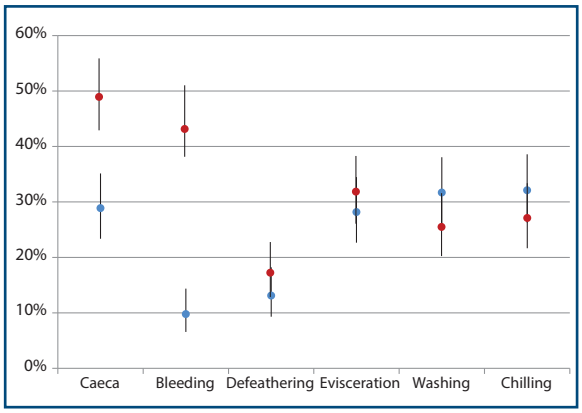

Figure 2. C. coli *(blue dots) and* C. jejuni *(red dots): prevalence (± C.L. 95%) of contamination of samples taken at slaughterhouse.*

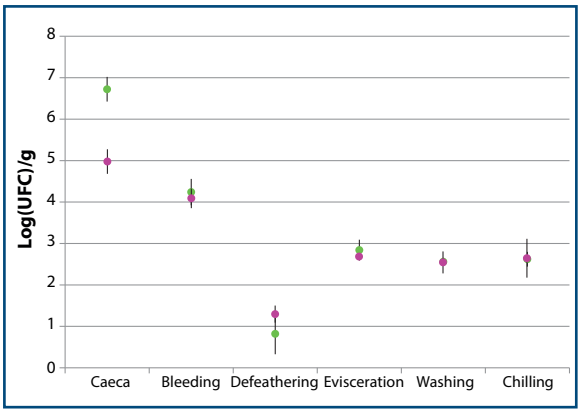

Figure 3. C. coli *(green dots) and* C. jejuni *(purple dots): levels of contamination (± C.L. 95%) of samples taken at slaughterhouse.*

C. coli showed significantly higher prevalence levels than of *C. jejuni* at washing ($p < 0.05$, $\chi^2 = 11.11$) and at chilling ($p < 0.05$, $\chi^2 = 9.26$). Figure 3 shows contamination levels of *C. jejuni* and *C. coli* in samples taken at slaughterhouse at different slaughtering stages. Concentrations of *C. coli* and *C. jejuni* on neck skin samples of carcasses collected after slaughter operations are shown in Table II and Table III, respectively. Statistically significant differences were found in *Campylobacter* concentration among the slaughtering stages ($p < 0.0001$; Kruskal Wallis test). Results of multiple comparisons performed with Dunn test highlighted that contamination levels of *C. coli* were significantly lower at defeathering [Mean: 1.54 Log (CFU)/g; SD: 0.91] and significantly higher at bleeding [Mean: 4.17 Log (CFU)/g; SD: 0.69] than at the other slaughtering stages ($p < 0.0001$; Kruskal Wallis test). Contamination levels of *C. jejuni* were significantly higher at bleeding [Mean: 4.08 Log (CFU)/g; SD: 1.05] than at the other slaughtering stages ($p < 0.0001$; Kruskal Wallis test). Details on *C. coli* and *C. jejuni* concentrations in samples of carcasses collected after the slaughter operations and on the results of the multiple comparisons performed are shown in Table II and Table III. As regards seasonality, concentration of *C. jejuni* in skin samples was found significantly higher in summer [Mean: 3.89 Log (CFU)/g; SD: 1.20] than in other sampling seasons ($p < 0.0001$; Kruskal Wallis test), while no statistically significant difference was found for *C. coli*.

In caeca samples no statistically significant difference was observed among contamination levels of seasons for *C. coli*, while a significant lower contamination

Table II. *The concentration of* C. coli *on samples of chicken carcasses collected after the slaughter operations.*

C. coli	Caeca	Bleeding*	Defeathering#	Evisceration	Washing	Chilling
Mean	6.81505115	4.170834346	1.540735312	2.919966832	2.574238328	2.664002739
Median	7.173475592	4.170915028	0.995635195	3.146128036	2.579783597	2.676091259
SD	1.112598967	0.694096517	0.906040819	0.793316118	0.296979432	0.630023145
95° perc	7.830002887	5.313414732	3.151232096	4.097512676	3.009558145	3.491361694
5° perc	4.044136728	3.090273684	0.995635195	0.995635195	2.301029996	2

*Contamination levels significantly higher ($p < 0.0001$); #Contamination levels significantly lower ($p < 0.0001$).

Table III. *The concentration of* C. jejuni *on samples of chicken carcasses collected after the slaughter operations.*

C. jejuni	Caeca	Bleeding*	Defeathering#	Evisceration#	Washing#	Chilling#
Mean	5.374044013	4.078751274	1.831685688	2.787108632	2.545574554	2.76650184
Median	5.627636253	3.954215699	0.995635195	2.903089987	2.544068044	3
SD	1.613333167	1.047516471	1.014431325	0.778875511	0.278445066	0.757715035
95° perc	7.661623156	5.946502352	3.538718726	3.678379134	3.004967555	3.614852317
5° perc	2.903089987	2.618264014	0.995635195	0.995635195	2.176091259	0.995635195

*Contamination levels significantly higher ($p < 0.0001$); #Contamination levels significantly lower ($p < 0.0001$).

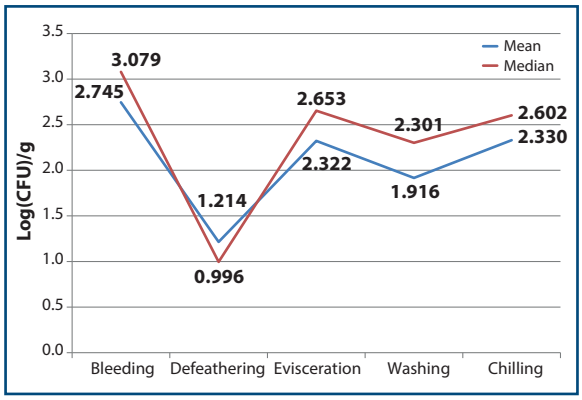

Figure 4. *Contamination levels of skin samples taken at slaughterhouse from the same animal.*

($p < 0.05$; Kruskal Wallis test) was detected in spring for *C. jejuni* [Mean: 4.30 Log (CFU)/g; SD: 0.77]. Significant differences between contamination levels of *C. coli* and *C. jejuni* along the slaughtering stages were found only for caeca samples with higher level of *C. jejuni* ($p < 0.05$; Fisher F-test).

As regards *Campylobacter* concentration in the same animal during the slaughtering chain, *Campylobacter* concentration was found significantly different among each sampling point ($p < 0.0001$; Friedman test). *Campylobacter* concentration was significantly lower at defeathering than at evisceration, washing and chilling, and significantly lower also at washing than at chilling stages ($p < 0.0001$; Nemenyi test). Figure 4 shows mean and median contamination levels found in skin samples of the same animal along the slaughtering chain.

PFGE

PFGE analysis with SmaI restriction enzyme of 367 *C. jejuni* isolates in caeca and in the phases of the slaughter process resulted in a total of 103 pulsotypes (with 100% similarity). PFGE types of *C. jejuni* strains isolated at the various stages of the slaughter process vary from 1 to 6 pulsotypes per cycle. Three PFGE types (31, 35 and 75) were always present in different product of all batches and farms, while six PFGE types (41, 53, 75, 82, 85 and 86) were present in all slaughtering stages (Table IV). For each slaughter batch, up to four different *C. jejuni* pulsotypes were found at slaughterhouse.

Cluster analysis of 273 *C. coli* isolates showed 70 distinct pulsotypes (with 100% similarity) with none of the PFGE types overlapping between the two farms. In particular, PFGE types of *C. coli* strains isolated at the different stages of the slaughter line

Table IV. *PFGE pulsotypes of* C. jejuni *strains isolated at different stages of the slaughter process and at farm and sampling season.*

Major pulsotypes (100% similarity)	Isolates (No.)	Source	Farm	Sampling season
26	4	Washing, Evisceration	B	Autumn
29	14	Defeathering, Evisceration, Washing, Chilling	B	Autumn
30	6	Evisceration, Washing, Chilling	B	Autumn
31	15	Defeathering, Evisceration, Washing, Chilling	B	Autumn
		Washing	C	Spring
35	14	Chilling	B	Autumn
		Washing	C	Winter
		Slaughtering bleeding, Evisceration, Washing	C	Spring
41	10	Slaughtering bleeding, Washing, Chilling	B	Summer
43	4	Slaughtering bleeding, Evisceration	C	Spring
53	25	Slaughtering bleeding, Evisceration, Chilling	A	Spring
68	10	Slaughtering bleeding, Evisceration, Washing, Caeca	A	Winter
72	2	Slaughtering bleeding, Evisceration	B	Summer
75	24	Defeathering	A	Summer
		Slaughtering bleeding, Evisceration, Washing	A	Winter
		Slaughtering bleeding, Evisceration, Washing, Chilling	B	Summer
77	2	Chilling, Defeathering	A	Summer
82	16	Slaughtering bleeding, Evisceration, Washing, Chilling	B	Summer
85	10	Slaughtering bleeding, Defeathering, Evisceration, Washing Chilling	C	Winter
86	32	Slaughtering bleeding, Defeathering, Chilling	C	Winter
87	10	Defeathering, Evisceration	C	Winter
93	2	Slaughtering bleeding, Defeathering	A	Summer
103	9	Slaughtering bleeding, Defeathering	B	Autumn

Table V. *PFGE pulsotypes of* C. coli *strains isolated at different stages of the slaughter process and at farm and sampling season.*

Major pulsotypes (100% similarity)	Isolates (No.)	Source	Farm	Sampling season
9	6	Defeathering, Washing, Chilling	A	Spring
10	2	Chilling, Evisceration	A	Spring
15	3	Defeathering, Washing	A	Autumn
17	7	Evisceration, Chilling	A	Autumn
18	18	Slaughtering bleeding, Washing, chilling	A	Autumn
24	18	Defeathering, Evisceration, Washing, Chilling	A	Winter
33	4	Evisceration, Washing, Chilling	C	Spring
41	6	Evisceration, Washing	A	Summer
43	3	Evisceration, Washing	A	Summer
44	36	Slaughtering bleeding, Evisceration, Washing, Chilling	A	Summer
70	42	Slaughtering bleeding, Defeathering, Evisceration, Washing, Chilling	A	Spring

vary from one to three pulsotypes for each batch and only three PFGE types (18, 44 and 70) were isolated at all slaughtering stages (Table V). Besides, for *C. coli* slaughter batches showed a multiple number of PFGE types, up to three, with the exception of four carcasses from farm C having a single pulsotype identified in all slaughtering stages.

Antimicrobial resistance

The results of MIC and antimicrobial resistance revealed that 92.0% and 93.8% of the isolates from caeca were resistant to quinolones and fluoroquinolones (NAL and Cip), respectively. The 39.3% of the strains showed resistance to tetracycline, the 13.4% to erythromycin, and few strains resulted resistant to other antimicrobials such as chloramphenicol (1.8%) and streptomycin (0.9%). None of the isolates tested was resistant or sensitive to gentamicin. Moreover, resistance to erythromycin was more frequent in *C. coli* (27.8%) compared to *C. jejuni* (6.6%) isolates ($p < 0.05$; χ^2 test), whereas these differences were not observed for the remaining antimicrobial substances.

The highest level of resistance was observed to NAL and Cip, for *Campylobacter* isolates from carcasses. In detail, the 90.0% and 90.6% of the strains were resistant to fluoroquinolones and quinolones, the 64.7% were resistant to tetracycline, and the 31.9% were resistant to erythromycin. The 99% of strains were susceptible to chloramphenicol, streptomycin gentamicin antimicrobials. Also for the strains isolated from carcasses, resistance to erythromycin was more frequent in *C. coli* isolates (44.0%) compared to *C. jejuni* (13.2%) ($p < 0.05$; χ^2 test).

Discussion

The prevention and control of *Campylobacter* colonisation in broiler flocks is an important goal for the reduction of campylobacteriosis in humans. To date, the mechanisms underlying *Campylobacter* colonization of farmed broiler flocks and contamination of carcasses during slaughtering have not been fully clarified yet. Investments in research are fundamental to improve the knowledge of the physiology, ecology, metabolism and colonisation mechanisms of *C. jejuni* and *C. coli* in poultry and their surviving capacity in the environment. In addition, although the role of *C. jejuni* contamination in broiler meat has been extensively studied in many European countries, the importance of *C. coli* in these food products has been not fully investigated yet. In particular, while the contribution of *C. jejuni* to the burden of human illness, through the consumption of raw or undercooked broiler meat, is well known, the same cannot be stated for *C. coli*.

Currently, in the EU it is generally considered that, given food regulations (EU 2017) precluding the use of antimicrobial treatments on carcasses (such as hyper chlorination), the most effective intervention strategy is to prevent or reduce flock colonisation at the farm level.

The results obtained at farm confirm the high level of prevalence already detected by other previous studies (Allen *et al.* 2007, Di Giannatale *et al.* 2010, Hadžiabdić *et al.* 2013, Henry *et al.* 2011, Hue *et al.* 2010, Rosenquist *et al.* 2006, Thakur *et al.* 2013). Regarding environmental samples, feed, water and pests, our results, however, are not in line with the findings of other studies (Evans *et al.* 2000, Bull *et al.* 2006) in which the isolation of *Campylobacter* was frequently obtained from feed, water in the drinkers and litter samples. In our research, the environmental samples and those taken from the feed, water and pests resulted all negative for *Campylobacter* detection, with the exception of one sample of water lake, which could suggest a possible introduction of contamination through the use of this water source for animal drinking.

Many studies are available on the different behaviour of *Campylobacter* species in farms and environment suggesting also a possible contribution of broiler farms to the aquatic environmental *Campylobacter* load (Schets *et al.* 2017). In our study, the comparison among *C. jejuni* and *C. coli* seems suggesting a different seasonal behaviour of *C. jejuni* with respect to *C. coli* in chickens, as shown in Figure 1. Moreover, it is noteworthy that whereas a clear seasonality of *Campylobacter* spp. contamination, with a highest risk from July to September, was observed in many EU member countries, this pattern was not recognised in Italy (EFSA 2010c). The increase of *Campylobacter* spp. contamination in broiler flocks during summer was frequently associated with the possible role of flies in spreading the infection within and between farms, especially in northern countries (Hald 2004). At southern latitudes, like in Italy, the temperatures conditions could be favourable for the presence of flies and other insects all around the year, thus lacking a clear seasonality in *Campylobacter* spp. contamination.

A recent report comparing different types of samples from broiler flocks at farm, showed a greater level of genetic diversity in strains isolated from neck skin and caeca samples than in chicken meat (Ugarte *et al.* 2015). Other studies investigating the genetic diversity of *Campylobacter* concluded that *Campylobacter* concentration increases from farm to slaughter, suggesting also that the full diversity of *Campylobacter* genotypes found at slaughter could be also the result of cross-contamination during the slaughtering process (Colles *et al.* 2010).

Commercial broiler farms are an important ecological niche for a wide variety of *Campylobacter* genotypes, thus confirming the complexity of the population structure of these organisms in broiler production and in the chicken food chain. Therefore, it is very important to improve sampling strategies with the aim of investigating *Campylobacter* structure population in broiler production (Vidal *et al.* 2016).

To our knowledge, this is the first extended study in which the sources of cross-contamination in a poultry slaughterhouse were studied by PFGE in Italy: we used PFGE to check the traceability of flock specific strains along the entire processing chain. With regard to species difficult to quantify, isolate, or distinguish, such as *Campylobacter* spp., PFGE is an important technique enabling research on the entire food supply chain as well as the tracing and estimation of the same strain responsible for contamination, from the raising of the animal to the foodborne illness in a human (Frasao *et al.* 2017). Even if PFGE is not the method of choice for molecular typing of *Campylobacter*, this technique is widely used for obtaining a clear comparison of genomic relationships among bacterial isolates, with the ability to correlate isolated microorganisms from different sites and samples (Frasao *et al.* 2017). Typing of *Campylobacter* strains isolated from pigs, poultry, turkey, sheep, and lambs by PFGE has been widely described (Silva *et al.* 2016, Lahti *et al.* 2017). In particular, Silva and colleagues found that *Campylobacter* clones belong to poultry flocks to indicate endemic strains with horizontal transmission among birds, and that the genetic profile associated with different farms suggested different sources of contamination (Silva *et al.* 2016).

PFGE results showed a high genetic heterogeneity of *Campylobacter* population: the same flocks were colonized by more genotypes. Some clones recovered at the early stages of the production chain were not recovered at the later stages, while other clones were predominant in individual breeding cycles and were present in all production chain stages (pulsotype 85 for *C. jejuni* or pulsotype 70 for *C. coli*), confirming the traceability of flock specific strains along the entire processing chain. Every season would seem characterised by different genetic sub-populations of strains of *Campylobacter*. However, other clones were identified within the same farm in more than one season (pulsotype 35 or 75 for *C. jejuni*), suggesting the persistence of these genotypes in the environment. These results are consistent with a study of Peyrat and colleagues showing the existence of *C. jejuni* and *C. coli* clones particularly able to adapt and survive overnight on the surfaces of slaughterhouse equipment after cleaning and disinfection (Peyrat *et al.* 2009).

On the contrary, the presence of the same clones of *C. jejuni* and *C. coli* in different herds (pulsotypes 31 for *C. jejuni*), spatially distant, might suggest a cross-contamination linked to the operators or the means used, favouring the recirculation of these strains among farms. Cross-contamination of carcasses from poultry coming from different flocks but slaughtered at same slaughterhouse seems, therefore, to be unavoidable.

The prevalence and contamination levels observed in carcasses confirm the deep influence of slaughtering operations to the final contamination levels. After a clear decrease of prevalence and contamination levels between bleeding and defeathering, a significant increase was observed after the evisceration stage. This trend may be related to the contamination occurred in case of intestine ruptures at the evisceration stage and supported by cross-contamination due to the strict contact among the carcasses during the slaughtering chain. The reduction of prevalence and contamination levels in skin samples taken after defeathering may be due to the effect of heat treatment after scalding (around 55 °C) (Bolder 2002), and to the abrasive

action caused by machines that remove microbial slide on the chicken skin.

The favourable effect in reducing the level of contamination by the exposure to low temperatures and the dehydration of the carcasses surface during the transit through the chilling tunnel was not confirmed in our study unlike other researches (Guerin *et al.* 2011).

The comparison among *C. jejuni* and *C. coli* concentrations in skin samples taken at slaughterhouse during seasons suggested a different behaviour of these organisms: *C. jejuni* seems to be significantly higher in summer, while *C. coli* concentration was not significantly different among seasons. An opposite prevalence trend among *C. jejuni* and *C. coli* was found after evisceration stage, as shown (Figure 2). *C. coli* showed significantly higher prevalence of contamination than of *C. jejuni* at washing and at chilling. This finding could suggest a greater resistance of *C. coli* than *C. jejuni* at lower temperatures, although no statistically significant difference was found among contamination levels of *C. coli* and of *C. jejuni* at washing and at chilling (Figure 3). In general, it is rather difficult to interpret these apparent differences, especially considering the microclimatic conditions in slaughterhouses, which are roughly constant and standardised. However, all these differences might suggest different mechanisms of persistence or survival capacities between *C. jejuni* and *C. coli* in the slaughtering environment, which should be more in depth investigated.

As regards antimicrobial resistance results, a significant increase in resistance to ciprofloxacin, nalidixic acid and tetracycline has been observed in this study. Erythromycin resistance is significantly more frequent in *Campylobacter coli* (27.8%) compared to *Campylobacter jejuni* (6.6%) in caeca isolates ($p < 0.05$; χ^2 test), and in *Campylobacter coli* isolates from chicken carcasses (44.0%) compared to *Campylobacter jejuni* (13.2%) ($p < 0.05$; χ^2 test). According to the other studies, high levels of resistance to tetracycline and ciprofloxacin are frequently reported in both the species (Ge *et al.* 2013, Rozynek *et al.* 2008). *C. coli* is usually more resistant to erythromycin than *C. jejuni*, although resistance in *C. jejuni* has been increasing (Wang *et al.* 2013).

Our study demonstrates the significant diffusion of *Campylobacter* infection in broilers in a typical breeding and slaughtering production context and the high genetic variability of the bacterium. To reduce cross contamination of *Campylobacter* flocks with persistent clones during the slaughtering process, efficient hygiene measures are needed. The results of this study provide more information for the definition of proper control options at slaughterhouses and new insight about the possible different behaviour of *C. coli* in comparison of *C. jejuni*.

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