Prevalence, phenotypic and genetic diversity of Campylobacter *in poultry fresh meat and poultry products on retail sale in Tuscany (Italy)*

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Keywords
Antimicrobial resistance, *Campylobacter*, Pulsed field gel-electrophoresis, Poultry meat, Poultry ready-to-cook products, Retail.

Summary
In this study, the prevalence of *Campylobacter* spp. in poultry fresh meat and ready-to-cook products was evaluated. Seventy-three samples were collected at retail level from supermarkets and discount stores, obtaining 61.6% positivity. Of 133 *Campylobacter* isolates, 86 strains (*Campylobacter coli*, 58.1% and *Campylobacter jejuni*, 41.9%) were selected for characterisation on the basis of their *Sma*I and *kpn*I pulsed field gel-electrophoresis (PFGE) profiles, to exclude clonal replicates. Campylobacters resulted highly resistant to tetracycline, ciprofloxacin, and nalidixic acid (79.1%, 72.1% and 65.1%, respectively); 50% of *C. coli* and 13.9% of *C. jejuni* were resistant to ciprofloxacin and erythromycin, the most important antimicrobials for human campylobacteriosis therapy. Five *C. coli* were resistant to 5/7 of the tested antimicrobials. HS4c was the prevailing *C. jejuni* serotype group (22.3%), whereas 8 other serotypes were identified in low percentages. *Sma*I and *kpn*I profiles showed a wide variability. The survey showed a high *Campylobacter* contamination of poultry meat and poultry products at retail level in Tuscany, Italy. A wide strains' heterogeneity and a remarkable level of strains' antimicrobial resistance have been reported, confirming the need for an improvement of specific preventive measures along the production chain.

Studio della prevalenza e della diversità fenotipica e genotipica di Campylobacter *isolati da carne e prodotti* ready-to-cook *di pollame commercializzati in Toscana (Italia)*

Parole chiave
Antibiotico-resistenza, *Campylobacter*, Carni di pollame, Prodotti di pollame *ready-to-cook*, Elettroforesi pulsata, Vendita al dettaglio.

Riassunto
Oggetto dello studio è stata la determinazione della prevalenza di *Campylobacter* termotolleranti in 73 campioni di carni di pollame e prodotti *ready-to-cook*, reperiti in Toscana a livello di grande distribuzione organizzata. Inoltre, su 133 isolati di *Campylobacter*, 86 ceppi (*C. coli*, 58,1% e *C. jejuni*, 41,9%), scelti sulla base dei profili di restrizione *Sma*I e *kpn*I in elettroforesi pulsata (PFGE) per escludere la presenza di repliche dello stesso ceppo, sono stati sottoposti a caratterizzazione. È stata ottenuta una prevalenza del 61,6% sul totale dei campioni (63,8% per le carni e 57,7% per i prodotti). I valori più elevati di antibiotico-resistenza sono stati riscontrati verso tetraciclina (79,1%) e chinoloni (72,1% per la ciprofloxacina e 65,1% per l'acido nalidixico). Il 50% dei *C. coli* ed il 13.9% dei *C. jejuni* è risultato resistente sia alla ciprofloxacina che all'eritromicina. Cinque *C. coli* sono risultati resistenti a 5/7 diversi antibiotici. HS4c è risultato il sierotipo prevalente di *C. jejuni* (22,3%), mentre altri 8 sierotipi sono stati evidenziati in percentuali nettamente inferiori. Ampia variabilità è emersa anche a livello genotipico nei ceppi di entrambe le specie. I risultati ribadiscono l'importanza di intensificare interventi mirati di prevenzione lungo l'intera filiera produttiva e in particolare a livello di allevamenti avicoli.

Introduction

Campylobacter is known as a major foodborne pathogen, worldwide. In the European Union (EU) it is the most commonly reported gastrointestinal bacterial pathogen in humans; 214,779 confirmed cases have been registered in 2013, with a notification rate of 64.8 per 100,000 population (EFSA-ECDC 2015). Similarly, in the USA an estimated 1.3 million people are affected each year (CDC-NCEZID 2013). *Campylobacter jejuni* is the species responsible for the majority of human cases of campylobacteriosis, followed by *Campylobacter coli* and *Campylobacter lari* (EFSA-ECDC 2014). *Campylobacter* contamination is frequent in various foods of animal origin, the main reservoir being the gastrointestinal tract of birds and mammals (particularly poultry, cattle, pig, and sheep). Worldwide, poultry carcasses and meat are routinely contaminated with *Campylobacter*, with a wide range of reported prevalence rates, even close to 100% (Zhao *et al.* 2001, Borck and Petersen 2005, Atanassova *et al.* 2007, Moran *et al.* 2009, Di Giannatale *et al.* 2010, Adzitey *et al.* 2012, Rejab *et al.* 2012, Wieczorek *et al.* 2013). Broiler meat is considered to be the main source of human campylobacteriosis (Sheppard *et al.* 2009). In 2013, 31.4% of fresh broiler meat resulted positive for *Campylobacter* in the EU, with important variations among the Member States (EFSA-ECDC 2015). Mishandling of raw poultry meat, consumption of undercooked meat, and cross-contamination of raw poultry to other foods are well-known risk-factors for acquiring *Campylobacter* infections (Silva *et al.* 2011).

In more severe cases of human campylobacteriosis antibiotic therapy may be needed. The first choice antimicrobials are fluoroquinolones and macrolides (EFSA-ECDC 2014). For this reason, the increase of antimicrobial resistance of *C. jejuni* and *C. coli* is of great concern for human health. The use of antimicrobials in livestock animals for prevention and treatment of bacterial diseases has contributed to the spread of resistance in foodborne bacteria, which may determine human infection. Particularly, the use of enrofloxacin in poultry breeding has been related to the increased resistance to ciprofloxacin in campylobacters isolated from both animals and humans (Mc Dermott *et al.* 2002, Nelson *et al.* 2007). Moreover, it has been shown that *Campylobacter* strains from poultry meat represent a potential risk for humans also because they can harbour several virulence factors (Melo *et al.* 2013). Thus, there is a need for a constant monitoring of *Campylobacter* strains from poultry products, along side to their antimicrobial resistance pattern as well as to their diversity profile, so to widen information about the global distribution of subtypes and their epidemiological role. This work evaluates the prevalence of thermotolerant campylobacters in poultry meat and ready-to-cook products purchased in Tuscan (Italy) retail stores and investigates some of the main phenotypic and genetic diversity characteristics of the strains isolated during the survey.

Materials and methods

Samples

Seventy-three ready-to-cook poultry samples (fresh chicken meat n. 34, fresh turkey meat n. 9, fresh meat of other poultry species n. 4, chicken/turkey ready-to-cook products, mainly represented by hamburgers, sausages, and skewered meat pieces, n. 26) were purchased in Tuscan retail stores of 10 supermarkets and discount chains from September 2011 to June 2012.

For the detection of *Campylobacter* spp., the ISO 10272-1:2006 method was applied[1], using Preston *Campylobacter* Selective Enrichment Broth, consisting of Nutrient Broth n° 2 (Oxoid, Basingstoke, UK) with the addition of Preston *Campylobacter* Selective Supplement, *Campylobacter* Growth Supplement and 5% of laked horse blood (Oxoid, Basingstoke, UK), instead of Bolton broth. For the inoculation phase, modified according to the method provided by Steele and McDermott (Steele and McDermott 1984), 0.3 ml of the enrichment broth were dispensed on cellulose membrane filters (0.45 µm pore size, Sigma Aldrich, Milan, Italy), placed on the surface of the agar plates required by the ISO standard and left for 45 minutes. The membranes were then removed, and the filtrate was spread on the plate surface. Colonies suspected of being *Campylobacter* (on average 4 per positive sample) were picked and subcultured to obtain monocultures. Isolates which resulted either difficult to purify or not fully viable and culturable were not considered. The remaining isolates were submitted to preliminary identification at genus level, as follows. Cells morphology and motility were evaluated by microscope observation by emulsifying a suspension of the isolate in 0.1 ml of contrast stain (1:1 Gram's crystal violet and saline solution) (BAM 2001); oxidase determination (Oxidase strips, Oxoid, Basingstoke, UK) and growth trials at 42°C in aerobiosis and at 25°C in microaerobic atmosphere were also performed. The isolates were then tested with the rapid identification system O.B.I.S. CAMPY (Oxoid, Basingstoke, UK), to rule out the production of L-alanyl aminopeptidase.

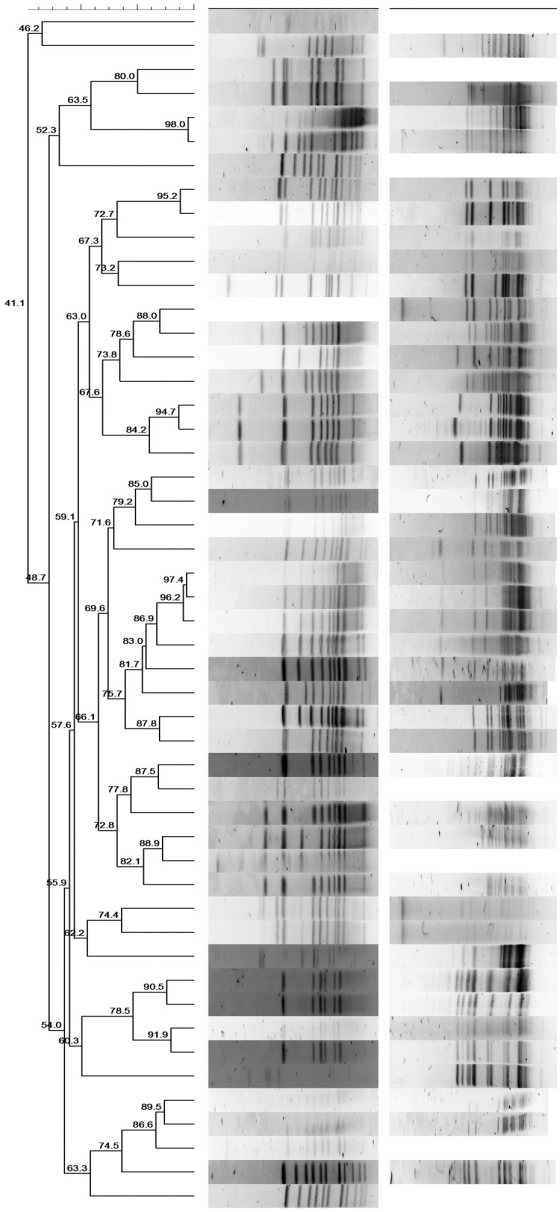
[1] International Organization for Standardisation (ISO). 2006. Microbiology of food and animal feeding stuffs – Horizontal method for detection and enumeration of *Campylobacter* spp. Part 1: Detection method. ISO 10272-1:2006.

Bacterial isolates

Isolates presumptively identified as *Campylobacter* spp. were stored at -70°C in Brain Heart Infusion Broth (Oxoid, Basingstoke, UK), added with 5% laked horse blood and 15% glycerol, for further identification and characterisation analyses.

Species identification

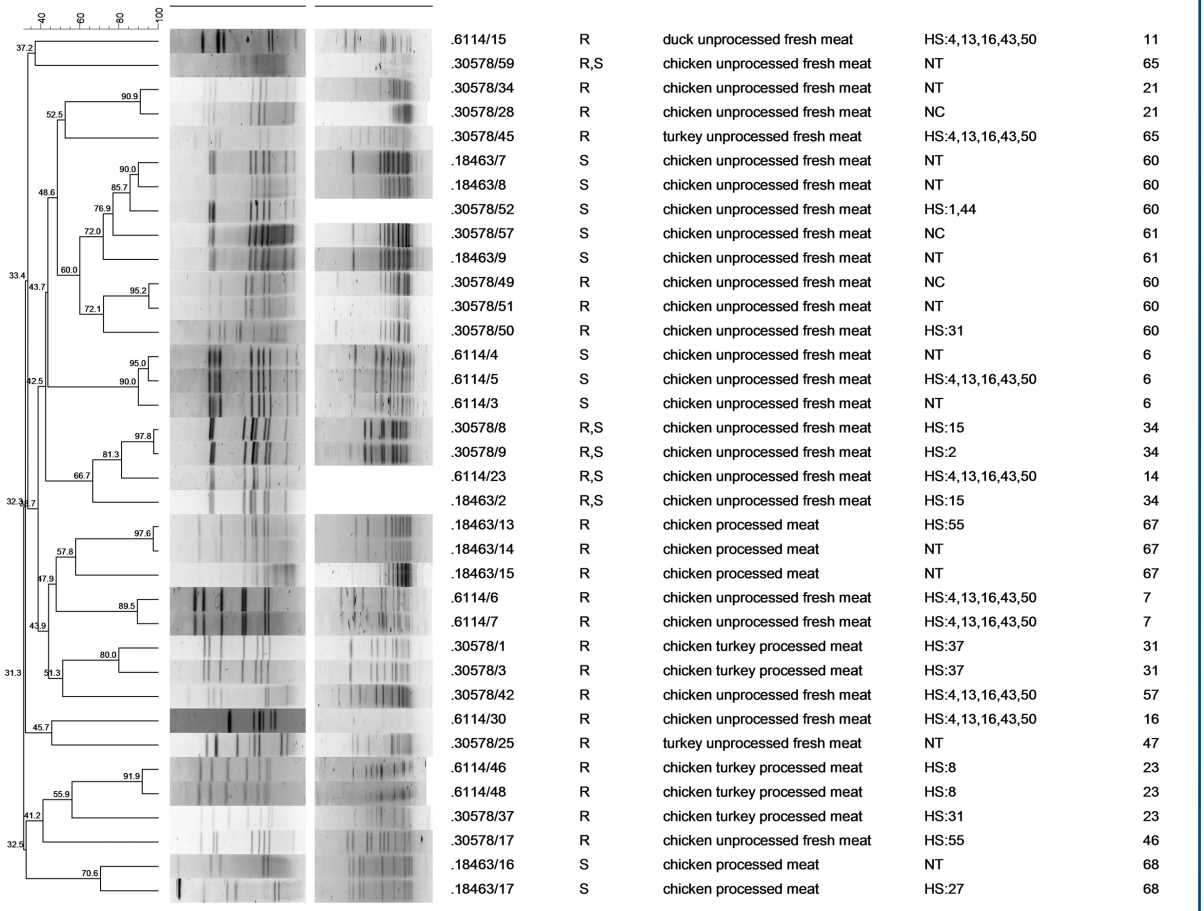
One hundred and thirty-three *Campylobacter* isolates were identified at species level by multiplex polymerase chain reaction (PCR) as described by Wang and colleagues (Wang *et al.* 2002). Strains used as positive controls were *Campylobacter coli* NCTC 11353, *Campylobacter fetus* ATCC 19438, *Campylobacter jejuni* ATCC 33291, *Campylobacter upsaliensis* NCTC 11541 and *Campylobacter lari* NCTC 11552.



S	QuR	Source	Source number
6114/43	R	guinea fowl unprocessed fresh meat	20
30578/58	S	chicken unprocessed fresh meat	61
18463/5	R	chicken processed meat	55
18463/11	S	chicken processed meat	66
30578/55	R	chicken unprocessed fresh meat	65
30578/56	R	chicken unprocessed fresh meat	65
30578/5	R	chicken unprocessed fresh meat	32
30578/6	R	turkey unprocessed fresh meat	33
30578/14	R	chicken turkey pork processed meat	37
30578/4	R	chicken unprocessed fresh meat	32
30578/13	R	chicken turkey pork processed meat	37
30578/15	R	chicken turkey pork processed meat	37
18463/22	R	chicken processed meat	72
30578/22	S	chicken unprocessed fresh meat	51
30578/16	R	chicken unprocessed fresh meat	45
18463/4	S	chicken unprocessed fresh meat	51
30578/46	S	turkey processed meat	59
30578/48	S	turkey processed meat	59
18463/6	S	chicken processed meat	59
6114/39	R	chicken unprocessed fresh meat	19
6114/26	R	chicken unprocessed fresh meat	14
30578/39	R	chicken unprocessed fresh meat	19
30578/12	R	chicken unprocessed fresh meat	35
30578/40	R	chicken unprocessed fresh meat	54
30578/26	R	chicken processed meat	48
30578/19	R	turkey unprocessed fresh meat	47
18463/3	R	chicken processed meat	48
6114/9	R	chicken unprocessed fresh meat	9
6114/50	S	turkey processed meat	28
18463/18	S	guinea fowl unprocessed fresh meat	69
18463/19	R	guinea fowl unprocessed fresh meat	69
6114/35	R	turkey processed meat	17
6114/44	R	chicken unprocessed fresh meat	18
6114/18	R	chicken unprocessed fresh meat	12
6114/19	R	chicken unprocessed fresh meat	12
6114/22	R	chicken unprocessed fresh meat	12
6114/20	R	chicken unprocessed fresh meat	12
18463/25	R	chicken processed meat	72
18463/26	R	chicken processed meat	72
6114/29	R	chicken unprocessed fresh meat	12
6114/33	S	chicken unprocessed fresh meat	16
6114/34	S	turkey processed meat	17
6114/36	S	chicken unprocessed fresh meat	18
6114/32	S	chicken unprocessed fresh meat	16
6114/31	S	chicken unprocessed fresh meat	16
6114/38	R, S	chicken unprocessed fresh meat	19
6114/40	R	guinea fowl unprocessed fresh meat	20
6114/37	R	chicken unprocessed fresh meat	19
6114/13	R	chicken unprocessed fresh meat	10
30578/11	R	chicken unprocessed fresh meat	35

S = sample; QuR = S: susceptibility to both tested quinolones, R: resistance to both tested quinolones, R/S: resistance to ciprofloxacin.
Some PFGE *kpn*I profiles were not available.

Figure 1. *Dendrogram representing relatedness among PFGE profiles of* SmaI *and* kpn*I digests, combined with resistance to quinolones, of* Campylobacter coli *strains selected for characterisation (n. 50), coming from 27 samples of poultry meat and ready-to-cook products purchased in Tuscan (Italy) retail stores from September 2011 to June 2012.*



S	QuR	Source	Serotype group	Source number
.6114/15	R	duck unprocessed fresh meat	HS:4,13,16,43,50	11
.30578/59	R,S	chicken unprocessed fresh meat	NT	65
.30578/34	R	chicken unprocessed fresh meat	NT	21
.30578/28	R	chicken unprocessed fresh meat	NC	21
.30578/45	R	turkey unprocessed fresh meat	HS:4,13,16,43,50	65
.18463/7	S	chicken unprocessed fresh meat	NT	60
.18463/8	S	chicken unprocessed fresh meat	NT	60
.30578/52	S	chicken unprocessed fresh meat	HS:1,44	60
.30578/57	S	chicken unprocessed fresh meat	NC	61
.18463/9	S	chicken unprocessed fresh meat	NT	61
.30578/49	R	chicken unprocessed fresh meat	NC	60
.30578/51	R	chicken unprocessed fresh meat	NT	60
.30578/50	R	chicken unprocessed fresh meat	HS:31	60
.6114/4	S	chicken unprocessed fresh meat	NT	6
.6114/5	S	chicken unprocessed fresh meat	HS:4,13,16,43,50	6
.6114/3	S	chicken unprocessed fresh meat	NT	6
.30578/8	R,S	chicken unprocessed fresh meat	HS:15	34
.30578/9	R,S	chicken unprocessed fresh meat	HS:2	34
.6114/23	R,S	chicken unprocessed fresh meat	HS:4,13,16,43,50	14
.18463/2	R,S	chicken unprocessed fresh meat	HS:15	34
.18463/13	R	chicken processed meat	HS:55	67
.18463/14	R	chicken processed meat	NT	67
.18463/15	R	chicken processed meat	NT	67
.6114/6	R	chicken unprocessed fresh meat	HS:4,13,16,43,50	7
.6114/7	R	chicken unprocessed fresh meat	HS:4,13,16,43,50	7
.30578/1	R	chicken turkey processed meat	HS:37	31
.30578/3	R	chicken turkey processed meat	HS:37	31
.30578/42	R	chicken unprocessed fresh meat	HS:4,13,16,43,50	57
.6114/30	R	chicken unprocessed fresh meat	HS:4,13,16,43,50	16
.30578/25	R	turkey unprocessed fresh meat	NT	47
.6114/46	R	chicken turkey processed meat	HS:8	23
.6114/48	R	chicken turkey processed meat	HS:8	23
.30578/37	R	chicken turkey processed meat	HS:31	23
.30578/17	R	chicken unprocessed fresh meat	HS:55	46
.18463/16	S	chicken processed meat	NT	68
.18463/17	S	chicken processed meat	HS:27	68

S = sample; QuR = S: susceptibility to both tested quinolones, R: resistance to both tested quinolones, R/S: resistance to ciprofloxacin.
Some PFGE *kpn*I profiles were not available.

Figure 2. *Dendrogram representing relatedness among PFGE profiles of* Sma*I and* kpn*I digests, combined with resistance to quinolones and Penner serogroups, of* Campylobacter jejuni *strains selected for characterisation (n. 36), coming from 17 samples of poultry meat and ready-to-cook products purchased in Tuscan (Italy) retail stores from September 2011 to June 2012.*

Pulsed field gel electrophoresis

Pulsed field gel electrophoresis (PFGE) of the 133 isolates was performed according to the instructions of the 2009 U.S.A. PulseNet protocol for *Campylobacter* (PulseNet U.S.A. 2009). Bacteria, previously identified by PCR, were subcultured onto Columbia agar and embedded in agarose blocks (Seakem Gold agarose, Lonza, Rockland, ME, USA). The blocks were then lysed, washed, digested with *Sma*I and *kpn*I restriction enzymes (Promega, Milan, Italy) and subjected to pulsed-field electrophoresis in 1% agarose gel (Seakem Gold agarose) for 18 hours (Chef Mapper® XA Pulsed Field Electrophoresis, Bio-rad, Hercules, CA, USA). *Salmonella* serovar Branderup H9812 was used as standard molecular weight size. After electrophoresis run, the gel was stained with Sybr Safe DNA gel stain (Invitrogen, Carlsbad, CA, USA) and photographed at transilluminator (Alpha Innotech, San Leandro, CA, USA). The image analysis was performed using the software Bionumerics v. 6.6 (Applied Maths NV, Sint Martens Latem, Belgium). Pair comparisons and cluster analyses were carried out using the Dice correlation coefficient and the unweighted pair group mathematical average (UPGMA) clustering algorithm. The optimization parameters and the position tolerance for band analysis were set at 1%. On the basis of PFGE results, in the case of isolates with indistinguishable PFGE profiles, belonging to the same clonal population, only 1 of them was submitted to further characterisation analyses. Thus, 86 *Campylobacter* strains were studied (Figure 1 and Figure 2).

Antimicrobial susceptibility

Susceptibility to antimicrobials of the 86 selected strains was evaluated with the microdilution method using the Sensititre automated system (TREK Diagnostic Systems, Venice, Italy). Colonies

were subcultured on Columbia agar for 24 hours and then seeded in Mueller Hinton Broth supplemented with blood (Oxoid, Basingstoke, UK) and dispensed into Eucamp microtiter plates (TREK Diagnostic Systems, Venice, Italy), containing known scalar concentrations of the following antibiotics: chloramphenicol (2-32 µg/ml), ciprofloxacin (0.06-4 µg/ml), erythromycin (0.5-32 µg/ml), gentamicin (0.12-16 µg/ml), nalidixic acid (2-64 µg/ml), streptomycin (1-16 µg/ml), and tetracycline (0.25-16 µg/ml). After inoculation, the plates were incubated at 42°C in microaerophilic atmosphere for 24 hours and then screened. *Campylobacter jejuni* strain NCTC 11351 was used as control. The strains were classified as resistant (R), intermediate (I) and susceptible (S) to the examined antimicrobials on the basis of Minimum Inhibitory Concentration (MIC) breakpoints, as reported in a previous study (Marotta *et al.* 2015). Chi square test was used to evaluate differences between resistance percentages of *C. jejuni* and *C. coli* strains to each antimicrobial. With regard to resistance to quinolones, of particular relevance in human campylobacteriosis therapy, the strains were registered as resistant (R) in case of resistance to both ciprofloxacin and nalidixic acid, susceptible (S) in case of receptiveness to both antimicrobials, and intermediate (RS or SR) in case of resistance to only 1 of the 2 considered quinolones (RS: resistant to ciprofloxacin, SR: resistant to nalidixic acid).

Serotyping of Campylobacter jejuni *isolates*

For *C. jejuni* strains, serotyping of heat-stable antigens was performed according to the classical Penner scheme (Penner & Hennessy 1980, Patton *et al.* 1985), based on passive haemagglutination, with a commercially available set of antisera (Denka-Seiken Co. Ltd., Tokyo, Japan). The absence of positive reaction was characterised as 'not typed' (NT).

Results

Forty-five out of 73 samples (61.6%) resulted positive for *Campylobacter* presence (63.8% of fresh meat and 57.7% of ready-to-cook products). Specifically, 70.6% of fresh chicken meat samples, 33.3% of fresh turkey meat samples, and 75% (3/4, guinea fowl and duck) of other species fresh meat samples tested positive. The 133 isolates, coming from 41 samples (28 fresh meat samples and 13 ready-to-cook samples), were identified as *C. coli* (n. 77, 57.9%) and *C. jejuni* (n. 56, 42.1%).

The 86 *Campylobacter* strains selected for characterisation (50 *C. coli*, 58.1% and 36 *C. jejuni*, 41.9%), coming from 39 samples, derived mostly (69.8%) from fresh meat (63.9% chicken and turkey, 5.8% other species). Five samples (12.8%) harboured both *C. coli* and *C. jejuni* strains.

Regarding the most important antimicrobials for human campylobacteriosis therapy, the examined *Campylobacter* strains showed an overall 72.1% and 34.9% resistance to ciprofloxacin and erythromycin, respectively (Table I). Particularly, 25/50 of *C. coli* strains (50%) and 5/36 of *C. jejuni* strains (13.9%) were resistant to both antimicrobials: *C. jejuni* strains were isolated from 3 samples (fresh duck meat, chicken breast, and chicken/turkey raw sausage); whereas *C. coli* came from 16 samples, mainly of unprocessed meat (12/16), among which 9 chicken meat samples, 2 guinea fowl samples, and 1 turkey meat sample. High resistance levels were registered also for tetracycline (79.1%) and nalidixic acid (65.1%); while the number of amynoglicosides-resistant campylobacters was low and all were susceptible to chloramphenicol. *Campylobacter coli* showed a higher percentage of resistance to all antimicrobials than *C. jejuni*, except for ciprofloxacin (*C. jejuni* 72.2% and *C. coli* 72%) and chloramphenicol (Figure 3). Although, a statistically significant difference ($P < 0.01$) was recorded only in the case of erythromycin and streptomycin. Resistance to both quinolones was registered in 65.1% of the tested campylobacters (70% of *C. coli* and 58.3% of *C. jejuni*), whereas 1 *C. coli* and 5 *C. jejuni* were resistant to ciprofloxacin, but susceptible to nalidixic acid (Figures 1 and 2). The antimicrobial patterns showed by the strains are reported in Table II. Six *C. coli* and 8 *C. jejuni* (16.3% of the total strains) were susceptible or intermediate to all antimicrobials. Thirty-one campylobacters out of 86 (36%), 5 of which were *C. jejuni*, were resistant to at least 4 antimicrobials. Five *C. coli*, isolated from 3 samples (2 fresh meat and 1 ready-to-cook product), showed

Table I. *Percentages of susceptibility/resistance of the* Campylobacter *strains selected for characterisation (n. 86).* The strains were isolated from 39/45 *Campylobacter*-positive samples of poultry meat and ready-to-cook products purchased in Tuscan (Italy) retail stores from September 2011 to June 2012.

	C	CIP	E	GE	NA	S	TE
S	86 (100)	24 (27.9)	42 (48.8)	83 (96.5)	30 (34.9)	71 (82.5)	16 (18.6)
I	0	0	14(16.3)	1 (1.2)	0	6 (7.0)	2 (2.3)
R	0	62 (72.1)	30 (34.9)	2 (2.3)	56 (65.1)	9 (10.5)	68 (79.1)

Results are expressed as strains number (percentage). S = susceptible; I = intermediate, R = resistant.
C = chloramphenicol; CIP = ciprofloxacin; E = erythromycin; GE = gentamicin; NA = nalidixic acid; S = streptomycin; TE = tetracycline.

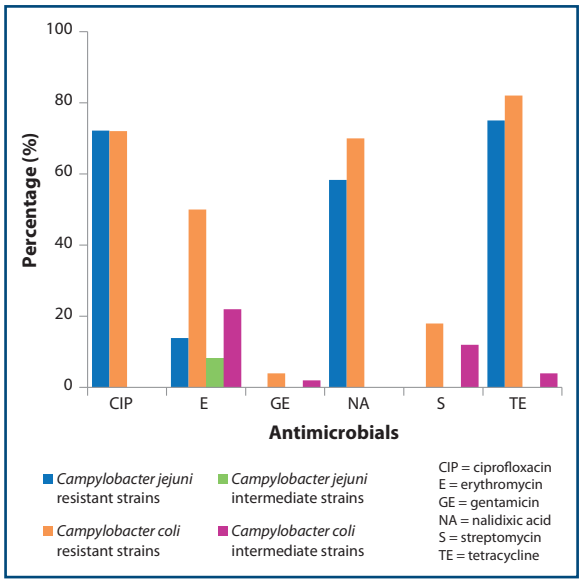


Figure 3. *Antimicrobial resistance profile of* Campylobacter *strains selected for characterisation (*Campylobacter coli *n. 50 and* Campylobacter jejuni *n. 36).* The strains were isolated from 39/45 *Campylobacter*-positive samples of poultry meat and ready-to-cook products purchased in Tuscan (Italy) retail stores from September 2011 to June 2012.

Table II. *Antimicrobials resistance patterns of the Campylobacter strains selected for characterisation (*Campylobacter coli *n. 50 and* Campylobacter jejuni *n. 36).* The strains were isolated from 39/45 *Campylobacter*-positive samples of poultry meat and ready-to-cook products purchased in Tuscan (Italy) retail stores from September 2011 to June 2012.

Resistance pattern	*Campylobacter coli* n. 50	*Campylobacter jejuni* n. 36
-	6	8
E	-	-
TE	6	2
CIP-NA	-	1
CIP-TE	1	5
S-TE	2	-
CIP-E-NA	-	2
CIP-NA-S	1	-
CIP-NA-TE	6	15
CIP-E-NA-TE	20	5
CIP-NA-S-TE	1	-
CIP-E-NA-S-TE	3	-
CIP-GE-NA-S-TE	2	-

CIP = ciprofloxacin; E = erythromycin, GE = gentamicin; NA = nalidixic acid; S = streptomycin; TE = tetracycline.

2 noteworthy multi-resistant profiles (ciprofloxacin, erythromycin, nalidixic acid, streptomycin, tetracycline; and ciprofloxacin, gentamycin, nalidixic acid, streptomycin, tetracycline).

The antigenic profile was determined only for *C. jejuni* strains, considering the primary epidemiological role of this species as causative agent of human campylobacteriosis. Using the classical Penner serotyping scheme, the most frequent serotype group was HS4c (HS 4, 13, 16, 43, 50, Group D) (22.3%), followed by HS8 (Group G), HS15 (Group L), HS31 (Group U), HS37 (Group Y), and HS55 (Group Z6) (5.6% each); while HS1/44 (Group A), HS2 (Group B) and HS27 (Group S) were sporadic. A high percentage of the strains resulted NT (33.3%), due to the absence of any positive serological reaction, or not classifiable (8.3%).

The molecular characterisation was conducted using 2 restriction enzymes (*Sma*I and *kpn*I) to increase the discriminatory power of PFGE. On the basis of molecular characterisation 50 and 36 pulsotypes were obtained for *C. coli* and *C. jejuni*, coming from 27 and 17 different samples, respectively. Considering a similarity higher than 60%, the combined analysis of the 2 enzymes, allowed for the distinction of the *C. coli* isolated from single samples (source number 65, 37, 51, 59, 19, 48, 69, 12, 72, and 16) in 10 clusters, with different similarity degrees ranging from 60.3% to 98% (Figure 1). Regarding *C. jejuni*, 8 clusters of strains isolated from single samples (source number 21, 60, 61, 6, 34, 7, 31, and 68), with similarity ranging from 60% to 97.8% (Figure 2), were obtained. Considering the heterogeneity within the samples, 9/39 positive samples (23.1%) harboured strains of the same species with PFGE profiles showing a similarity lower than 60% (Figure 1 and Figure 2).

An interesting genetic group was composed by 3 *C. coli* strains isolated from 3 samples of both processed and unprocessed meat, showing a PFGE similarity of 96.2%. Two other *C. coli* strains, isolated from 2 samples of fresh and processed meat, showed a 95.2% similarity (Figure 1). This could suggest a circulation of some genetic types along the production chain.

With regard to the phenotypical heterogeneity of strains derived from the same sample, in 17.6% (3/17) of *C. jejuni* positive samples strains belonging to different serogroups were isolated. Moreover, it is noteworthy that 30.8% (12/39) of the positive samples harboured strains of the same species with antimicrobial resistance patterns differing for 1-3 antimicrobials (not counting I-S and I-R as differences) (data not shown).

Discussion

From 2005 onward, campylobacteriosis has been the most commonly reported zoonosis in humans. According to the last EFSA report (EFSA-ECDC 2015), in 2013, as in previous years, broiler meat was the most frequently identified food vehicle of

human campylobacteriosis in EU, associated with 50% of strong evidence outbreaks. More generally, the *Campylobacter* contamination level of poultry meat is remarkable all over the world. According to the literature survey provided by Suzuki and Yamamoto (Suzuki and Yamamoto 2009), covering 2002-2007, most of developed and developing countries with available data showed a prevalence of *Campylobacter* contamination in retail poultry meats equal or higher than 50%. More recently, an overall *Campylobacter* prevalence of 85.1% in raw retail poultry meat has been reported in Northern Ireland (Moran *et al.* 2009), and 87.2% in raw chicken meat in Poland (Wieczorek *et al.* 2012), while a lower level of *Campylobacter* contamination (20.8%) in poultry meat collected at retail level has been reported in Estonia (Mäesaar *et al.* 2014). As for Italy, in the past years different levels of *Campylobacter* contamination have been reported: 81.3% in chicken meat in North-Eastern Italy (Pezzotti *et al.* 2003), 73.3% in 30 broiler meat samples sold in South-Eastern Italy (Parisi *et al.* 2007), 40.8% in raw chicken meat marketed in Abruzzo and Molise regions (Prencipe *et al.* 2007), 51% in chicken meat samples purchased in Campobasso (Molise, Italy) markets and butcher's shops (Sammarco *et al.* 2010). More recently, Nobile and colleagues examined 208 samples of chicken and turkey meat collected from butcher's shops in Catanzaro (Southern Italy), obtaining a 18.6% and 23.1% *Campylobacter* prevalence in chicken and turkey meat, respectively (Nobile *et al.* 2013).

In our survey, we obtained a prevalence of about 70% in fresh chicken meat, very similar to the one registered by Parisi and colleagues (Parisi *et al.* 2007). This result shows that the level of *Campylobacter* contamination remains high in Italy. In contrast with the study conducted by Nobile and colleagues (Nobile *et al.* 2013), who registered a higher *Campylobacter* presence in turkey meat than in chicken meat, we found that the prevalence in chicken meat was more than twice as much as the one in turkey meat. A lower contamination in turkey meat than in chicken meat has also been reported in other studies (Moran *et al.* 2009), and in the last European Food Safety Authority report on zoonoses (EFSA-ECDC 2015), which found that turkey meat is contaminated at moderate level.

It is noteworthy that in our survey a considerable percentage (about 58%) of chicken/turkey ready-to-cook products tested positive for *Campylobacter*. Even if it is known that the processing phases are usually able to determine some decrease in campylobacters counts in such products, quantitative data on their *Campylobacter* contamination level are scanty (Habib *et al.* 2008). Thus, they have to be considered as an additional possible vehicle of human campylobacteriosis. According to EFSA (EFSA-ECDC 2015), after broiler meat, "other mixed or unspecified poultry meats and products thereof" resulted the next most commonly implicated food vehicle in case of strong evidence outbreaks of human campylobacteriosis.

With regard to strains' characterisation, in the case of multiple strains with indistinguishable PFGE profiles, even if isolated from different samples, we decided to consider only 1 of such strains, to better focus on studying strain diversity. However, it has to be noted that in this way it is possible that strains with the same PFGE pulsotypes but with different phenotypical patterns were excluded.

As for the species identification of the examined campylobacters, we found that *C. coli* was the prevailing species. Usually, *C. jejuni* is the most frequently isolated species, but variable ratios of *C. coli* to *C. jejuni* have been reported (Suzuki and Yamamoto 2009). The most recently published Italian survey found that *C. coli* was the prevailing species, particularly in chicken meat (Nobile *et al.* 2013).

The strains' antimicrobial profile showed, as expected, high percentages of resistance to tetracycline and quinolones and *C. coli* was confirmed as being generally more resistant than *C. jejuni*, in accordance with the latest EFSA report on antimicrobial resistance in zoonotic bacteria (EFSA-ECDC 2014). Interestingly, an overall 34.9% of resistance to erythromycin was registered, with 50% (25/50, as shown in Table II) and 13.9% (5/36, Table II) of resistant *C. coli* and *C. jejuni*, respectively. Instead, in 2014 the European Food Safety Authority (EFSA-ECDC 2014) reported a 16.5% and 1.8% resistance to erythromycin in *C. coli* and *C. jejuni* from broiler meat on the basis of data from 8 and 6 Member States, respectively. Moreover, an important percentage of strains resulted resistant to both ciprofloxacin and erythromycin. Similarly, Sammarco and colleagues (Sammarco *et al.* 2010) observed 43.5% and 30% ciprofloxacin-erythromycin resistant *C. coli* and *C. jejuni*, respectively. Additionally, Nobile and colleagues (Nobile *et al.* 2013) reported that 20.9% of isolates was susceptible to both ciprofloxacin and erythromycin. Data from both studies are generally consistent with our results; however, differences may be due to different methodology (disk diffusion method).

Campylobacter jejuni strains were studied also with regard to their Penner serotypes distribution. The most prevailing serotype group was HS4c, which is, alongside to HS2 and HS1/44, one of the dominant serotypes of *C. jejuni* related to human cases of campylobacteriosis all-over the world (Pike *et al.* 2013). We found a 22.3% prevalence of this serotype group, this percentage is higher than the ones reported in similar studies on retail poultry products, where percentages of 13% have been noted (Saito *et al.* 2005, Nielsen *et al.* 2006). Nine serotype

groups were revealed in our survey, showing a wide variability of the studied *C. jejuni* strains.

Our PFGE typing results showed a similar strain variability for both *C. jejuni* and *C. coli*. A similar high genetic diversity has recently been reported for Italian *Campylobacter* strains of animal, food, and human origin, using PFGE *Sma*I typing (Di Giannatale *et al.* 2014). Moreover, we obtained different phenotypical and genotypical profiles even in strains isolated from the same sample. At the same time, the presence of at least 1 genetic group, comprising *C. coli* strains with high PFGE similarity, recovered from different samples suggests a wide circulation of some *Campylobacter* pulsotypes along the poultry production chain. This result confirms, although with a limited number of studied strains, the epidemiological relevance of this genetic characterisation, which provides a contribution to identify prevailing *Campylobacter* subtypes in the poultry food chain.

In conclusion, this study has confirmed the high prevalence of pathogenic *Campylobacter* not only in fresh poultry meat, but also in ready-to-cook products that commonly enter the consumers' kitchens. Moreover, it has revealed high antimicrobial resistance percentages in campylobacters isolated from such food sources. In particular, in the case of erythromycin, these percentages resulted remarkably higher than those reported by European Food Safety Authority/European Centre for Disease Prevention and Control (EFSA-ECDC 2014). Thus, there is a need for continuous and more incisive preventive measures to limit the spread of such resistances, paying particular attention to the antimicrobial utilization at poultry breeding level.

References

Adzitey F., Huda N. & Rahmat Ali G.R. 2012. Prevalence and antibiotic resistance of *Campylobacter, Salmonella*, and *L. monocytogenes* in ducks: a review. *Foodborne Pathog Dis*, **9** (6), 498-505.

Atanassova V., Reich F., Beckmann L. & Klein G. 2007. Prevalence of *Campylobacter* spp. in turkey meat from a slaughterhouse and in turkey meat retail products. *FEMS Immunol Med Microbiol*, **49**, 141-145.

Bacteriological Analytical Manual (BAM). 2001 (http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm072616.htm, accessed on 7 January 2016).

Borck B. & Petersen K. 2005. Pulsed-field gel electrophoresis types of *Campylobacter* spp. in Danish turkeys before and after slaughter. *Int J Food Microbiol*, **101**, 63-72.

Centers for Disease Control and Prevention - National Center for Emerging and Zoonotic Infectious Diseases (CDC-NCEZID). 2013 (www.cdc.gov/nczved/divisions/dfbmd/diseases/campylobacter/technical.html, accessed on 7 January 2016).

Di Giannatale E., Prencipe V., Colangeli P., Alessiani A., Barco L., Staffolani M., Tagliabue S., Grattarola C., Cerrone A., Costa A., Pisanu M., Santucci U., Iannitto G. & Migliorati G. 2010. Prevalence of thermotolerant *Campylobacter* in boiler flocks and broiler carcasses in Italy. *Vet Ital*, 46 (4), 415-423.

Di Giannatale E., Di Serafino G., Zilli K., Alessiani A., Sacchini L., Garofolo G., Aprea G. & Marotta F. 2014. Characterization of antimicrobial resistance patterns and detection of virulence genes in *Campylobacter* isolates in Italy. *Sensors*, **14**, 3308-3322.

European Food Safety Authority and European Centre for Disease Prevention and Control (EFSA-ECDC). 2014. The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2012. *EFSA Journal*, **12** (3), 3590 (http://www.efsa.europa.eu/it/efsajournal/pub/3590.htm, accessed on 7 January 2016).

European Food Safety Authority and European Centre for Disease Prevention and Control (EFSA-ECDC). 2015. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2013. *EFSA Journal*, **13** (1), 3991 (http://www.efsa.europa.eu/it/efsajournal/pub/3991.htm, accessed on 7 January 2016).

Habib I., Sampers I., Uyttendaele M., Berkvens D. & De Zutter L. 2008. Baseline data from a Belgium-wide survey of *Campylobacter* species contamination in chicken meat preparations and considerations for a reliable monitoring program. *Appl Environ Microbiol*, **74** (17), 5483-5489.

Mäesaar M., Praakle K., Meremäe K., Kramarenko T., Sõgel J., Viltrop A., Muutra K., Kovalenko K., Matt D., Hörman A., Hänninen M-L. & Roasto M. 2014. Prevalence and counts of *Campylobacter* spp. in poultry meat at retail level in Estonia. *Food Control*, **44**, 72-77.

Marotta F., Garofolo G., Di Donato G., Aprea G., Platone I., Cianciavicchia S., Alessiani A. & Di Giannatale E. 2015. Population diversity of *Campylobacter jejuni* in poultry and its dynamic of contamination in chicken meat. *BioMed Research International*, article ID 859845. http://dx.doi.org/10.1155/2015/859845.

McDermott P.F., Bodeis S.M., English L.L., White D.G., Walker R.D., Zhao S., Simjee S. & Wagner D.D. 2002. Ciprofloxacin resistance in *Campylobacter jejuni* evolves rapidly in chickens treated with fluoroquinolones. *J Infect Dis*, **185** (6), 837-840.

Meldrum R.J. & Wilson I.G. 2007. *Salmonella* and *Campylobacter* in United Kingdom retail raw chicken. *J Food Prot*, **70**, 1937-1939.

Melo R.T., Nalevaiko P.C., Mendonça E.P., Borges L.W., Fonseca B.B., Beletti M.E. & Rossi D.A. 2013. *Campylobacter jejuni* strains isolated from chicken meat harbour several virulence factors and represent a potential risk to humans. *Food Control*, **33**, 227-231.

Moran L., Scates P. & Madden R.H. 2009. Prevalence of *Campylobacter* spp. in raw retail poultry in Northern Ireland. *J Food Prot*, **9**, 1830-1835.

Nelson J.M., Chiller T.M., Powers J.H. & Angulo F.J. 2007. Fluoroquinolone-resistant *Campylobacter* species and the withdrawal of fluoroquinolones from use in poultry: a public health success story. *Clin Infect Dis*, **44** (7), 977-980.

Nielsen E.M., Fussing V., Engberg J., Nielsen N.L. & Neimann J. 2006. Most *Campylobacter* subtypes from sporadic infections can be found in retail poultry products and food animals. *Epidemiol Infect*, **134**, 758-767.

Nobile C.G.A., Costantino R., Bianco A., Pileggi C. & Pavia M. 2013. Prevalence and pattern of antibiotic resistance of *Campylobacter* spp. in poultry meat in Southern Italy. *Food Control*, **32** (2), 715-718.

Parisi A., Lanzilotta S.G., Addante N., Normanno G., Di Modugno G., Dambrosio A. & Montagna C.O. 2007. Prevalence, molecular characterization and antimicrobial resistance of thermophilic *Campylobacter* isolates from cattle, hens, broilers and broiler meat in South-eastern Italy. *Vet Res Comm*, **31**, 113-123.

Patton C.M., Barrett T.J. & Morris K.G. 1985. Comparation of the Penner and Lior methods for serotyping *Campylobacter. J Clin Microbiol*, **22** (4), 558-565.

Penner J.L. & Hennessy J.N. 1980. Passive hemagglutination technique for serotyping *Campylobacter fetus* subsp. *jejuni* on the basis of soluble heat-stable antigens. *J Clin Microbiol*, **12**, 732-737.

Pezzotti G., Serafin A., Luzzi I., Mioni R., Milan M. & Perin R. 2003. Occurrence and resistance to antibiotics of *Campylobacter jejuni* and *Campylobacter coli* in animals and meat in northeastern Italy. *Int J Food Microbiol*, **82**, 281-287.

Pike B.L., Guerry P. & Poly F. 2013. Global distribution of *Campylobacter jejuni* Penner serotypes: a systematic review. *PLoS ONE*, **8** (6), e67375.

Prencipe V., Parisciani G., Calistri P., Caporale C.M., Iannitto G., Morelli D., Pomilio F., Prochowski D. & Migliorati G. 2007. Thermotolerant *Campylobacter* in poultry meat marketed in the Abruzzo and Molise regions of Italy: prevalence and contamination levels. *Vet Ital*, **43** (1), 167-174.

PulseNet U.S.A. – The National Molecular Subtyping Network for Foodborne Disease Surveillance. CDC. One-day (24-26 h) standardized laboratory protocol for molecular subtyping of *Campylobacter jejuni* by Pulsed Field Gel Electrophoresis (PFGE). August 2009.

Rejab S.B., Zessin K.H. & Patchanee P. 2012. *Campylobacter* in chicken carcasses and slaughterhouses in Malaysia. *Southeast Asian J Trop Med Public Health*, **43** (1), 96-104.

Saito S., Yatsuyanagi J., Harata S., Ito Y., Shinagawa K., Suzuki N., Amano K. & Enomoto K. 2005. *Campylobacter jejuni* isolated from retail poultry meat, bovine feces and bile, and human diarrhea samples in Japan: comparison of serotypes and genotypes. *FEMS Immunol Med Microbiol*, **45**, 311-319.

Sammarco M.L., Ripabelli G., Fanelli I., Grasso G.M. & Tamburro M. 2010. Prevalence and biomolecular characterization of *Campylobacter* spp. isolated from retail meat. *J Food Prot*, **73** (4), 720-728.

Sheppard S.K., Dallas J.F., Strachan N.J.C., MacRae M., McCarthy N.D., Wilson D.J., Gormley F.J., Falush D., Ogden I.D., Maiden M.C.J. & Forbes K.J. 2009. *Campylobacter* genotyping to determine the source of human infection. *Clin Infect Dis*, **48**, 1072-1078.

Silva J., Leite D., Fernandes M., Mena C., Gibbs P.A. & Teixeira P. 2011. *Campylobacter* spp. as a foodborne pathogen: a review. *Front Microbiol*, **2**, 200. doi:10.3389/fmicb.2011.00200.

Steele T.W. & McDermott S.N. 1984. The use of membrane filters applied directly to the surface of agar plates for the isolation of *Campylobacter jejuni* from feces. *Pathology*, **16** (3), 263-265.

Suzuki H. & Yamamoto S. 2009. *Campylobacter* contamination in retail poultry meats and by-products in the world: literature survey. *J Vet Med Sci*, **71** (3), 255-261.

Thakur S., Zhao S., McDermott P.F., Harbottle H., Abbott J., English L., Gebreyes W.A. & White D.G. 2010. Antimicrobial resistance, virulence, and genotypic profile comparison of *Campylobacter jejuni* and *Campylobacter coli* isolated from humans and retail meats. *Foodborne Pathog Dis*, **7**, 1-10.

Wang G., Clark C.G., Taylor M.T., Pucknell C., Barton C., Price L., Woodward D.L. & Rodgers F.G. 2002. Colony multiplex PCR Assay for identification and differentiation of *Campylobacter jejuni, Campylobacter lari, Campylobacter upsaliensis, Campylobacter fetus* sub. *fetus. J Clin Microbiol*, **40**, 4744-4747.

Wieczorek K., Szewczyk R. & Osek J. 2012. Prevalence, antimicrobial resistance, and molecular characterization of *Campylobacter jejuni* and *C. coli* isolated from retail raw meat in Poland. *Veterinarni Medicina*, **57** (6), 293-299.

Wieczorek K., Kania I. & Osek J. 2013. Prevalence and antimicrobial resistance of *Campylobacter* spp. isolated from poultry carcasses in Poland. *J Food Prot*, **76** (8), 1451-1455.

Zhao C., Ge B., De Villena J., Sudler R., Yeh E., Zhao S., White D.G., Wagner D. & Meng J. 2001. Prevalence of *Campylobacter* spp., *Escherichia coli* and *Salmonella* serovars in retail chicken, turkey, pork, and beef from the Greater Washington, D.C., Area. *Appl Environ Microbiol*, **67** (12), 5431-5436.