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Department of Life and Environmental Sciences

Curriculum Biomolecular Sciences

Investigation of *Campylobacter* spp in broiler chickens from farm to retail

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CHAPTER 1: Introduction

1.1 Genus *Campylobacter*

1.1.1 Taxonomy

In 1886, Theodor Escherish identified a spiral shaped bacteria in stool sample from children with gastroenteritis disease called "cholera infantum". In 1906, two British veterinary surgeons reported the presence of "large numbers of a peculiar organisms" that were frequently isolated from pregnant ewes and which resembled as *Vibrio*-like bacteria, due to spiral morphology. In 1927, Smith and Orcutt found a group of bacteria in the feces of cattle with diarrhea. In 1931, Jones and colleagues attributed a relationship between dysentery in calves and infection with microaerophillic vibrios. Later the microorganism was called *Vibrio jejuni*. The firs well documented case of human *Campylobacter* infection occurred in Illinois in 1938. This caused a milk-borne outbreak of gastroenteritis, that infected 355 inmates in two adjacent state institutions. In 1944, Doyle isolated a similar vibrio from feces of swine with dysentery and classified them as *Vibrio coli*. In 1963 Sebal and Vèron proposed a new genus, *Campylobacter*, distinguishing from *Vibrio* spp, due to their low DNA base (low guanine and cytosine) composition, non fermentative metabolism and their microaerophillic grown requirements (Epps *et al*., 2013). The first *Campylobacter* isolated from human diarrhea was described by Dekeyser *et al*. in 1972. The *Campylobacter* genus belongs to family *Campylobacteriaceae*, the order *Campylobacterales*, in the epsilon subdivision of the Proteobacteria, called rRNA superfamily VI, which also includes *Arcobacter* and

Helicobacter (On *et al*., 2001). The genus *Campylobacter* currently comprises 26 species, 2 provisional species and 9 subspecies (Kaakoush *et al*., 2015). Over 80% and approximately 10% of the human cases are caused by *Campylobacter jejuni* and *Campylobacter coli*, respectively (EFSA, 2011b). There are two subspecies recognized within *C.jejuni*, *C.jejuni* subspecies *jejuni* and *C.jejuni* subspecies *doylei*. Strains of *C.doylei* differ from *C.jejuni* biochemically in its inability to reduce nitrate and growth at 42°C (subsp. *doylei* does not grow at 42°C). Subspecies *jejuni* is more frequently isolated than subspecies doylei (Epps *et al*., 2013).

1.1.2 Biology

The word "*Campylobacter*" is derived from the Greek word "campylo" which denotes to its morphological shape which is curved rod, spiral or "S" shaped morphology under the microscope. *Campylobacter* species are non spore-forming, are Gram-negative, small (0.2–0.9 μm wide and 0.2–5.0 μm long) and motile bacteria, with a single polar unsheathed flagellum at one or both ends. Most *Campylobacter* spp. are catalase and oxidase positive. These bacteria produce catalase to reduce the toxic product hydrogen peroxide (H_2O_2) created in their environment. *Campylobacter* are oxidase-positive because they contain cytochrome C, a compound that helps transfer electrons to the cytochrome oxidase complex (Eberle and Kiess, 2012). The bacteria, unlike other enteric pathogens, *Escherichia coli* and *Salmonella*, do not utilize exogenous glucose and mainly depends on the catabolism of amino-acids or tricarboxylic acid cycle intermediates; they can neither ferment nor oxidize carbohydrates (Jeon *et al*., 2010). The organisms grow quite slowly and they are essentially microaerophilic, requiring a lower concentration of oxygen and a higher carbon dioxide concentration (5% O_2, 10% CO_2 and 85% N_2) but some can also grow aerobically or anaerobically. Thermophilic *Campylobacter* species (*C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*,

C. helveticus) do not grow below 30°C (absence of could shock protein genes which play a role in low-temperature adaption) and have an optimal growth temperature at 42°C in microaerophilic conditions. Moreover, these microorganisms do not exhibit true thermophily (growth at 55°C or above). Generally, *C. jejuni* (hippurate - positive) can be differentiated from other *Campylobacter* species on the basis of the hydrolysis of hippurate. *Campylobacter coli* cannot hydrolize hippurate; however, the presence of hippuare - negative *C. jejuni* strains has been reported (Silva *et al*., 2011). Under unfavorable growth conditions, these microorganisms have the ability to change from the characteristic spiral shape in the exponential phase to a coccoid shape in the viable but non-culturable (VBNC) state (Thomas *et al*., 2002). A study showed that *Campylobacter* strains, isolated from the soil around the broilers house, may have been transformed into viable but non-cultivable forms (Fig. 1) and might have become cultivable after passing through the intestinal tract of chickens (Cappelier, 1997).

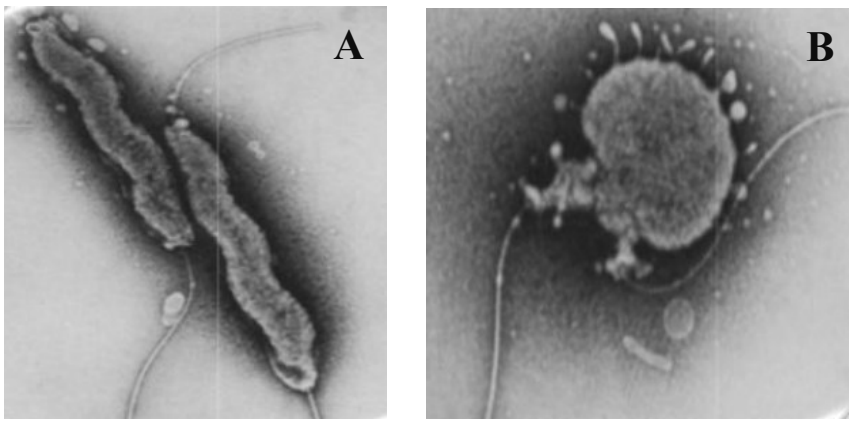


Figure 1: *Campylobacter jejuni* forms: a) spiral form; b) coccoid form (Pead, 1979)

Recently, the complete genome sequence of *C.jejuni*, in particulary *C.jejuni* NCTC11168, was characterized. *Campylobacter jejuni* has a circular chromosome of 1.641.481 base pairs (30,6% G+C) (small genome size), which is predicted to encode 1.654 proteins and 54 stable RNA species. One surprising feature of *C.jejuni* genome is the almost complete lack of repetitive DNA sequences; in fact there are only four repeated sequences within the entire genome. Moreover, the presence of hypervariable regions might be important in the survival of the organism (Parkhill *et al*., 2000).

The development of the disease involves adaptation of the bacteria to harsh gut environment, where they can survive and adhere to intestinal epithelial cells. Although *Campylobacters* are fragile and sensitive to environment and to a wide range to stress factors, they exhibits great flexibility in the adaptation to numerous hostile habitats including the gastrointestinal tract. This high adaptability is attributable to its genetically, metabolically and phenotypically diverse population structure and its ability to change in response to various challenges. *Campylobacter* proves highly mutable in response to antibiotic treatments and possesses eukaryote-like dual protein glycosylation systems, which modify flagella and other surface proteins with specific sugar structures (Rubinchik *et al*., 2012).

1.1.3 Epidemiology and typing

The numbers of cases of campylobacteriosis have increased in Europe. In 2014, *Campylobacter* continued to be the most commonly reported gastrointestinal bacterial pathogen in humans in the European Union (EU) since 2005 (EFSA and ECDC, 2015). The number of reported confirmed cases of human campylobacteriosis was 236.851 which registered an increase of 22.067 cases compared with 2013 (Fig. 2 and Tab. 1). The EU notification rate was 71.0 per 100.000 population in 2014, which showed an increase by 9.6% compared with 2013 (64.8 per 100.000 population) (EFSA and ECDC, 2015). In Italy, 1252 cases of human Campylobacteriosis were reported in 2014. However, the available data do not reflect the real number of cases (EFSA and ECDC, 2015) because in Italy the reporting system of infectious diseases does not differentiate between gastroenteritis caused by *Campylobacter* and gastroenteritis caused by the other agents listed in the National Legislation in Italy. Therefore, campylobacteriosis is not subject to statutory notification in

Italy and the only data available on these infections are those reported voluntarily by Enter- Net, the international network for the surveillance of human gastrointestinal infections (Di Giannatale *et al*., 2016).

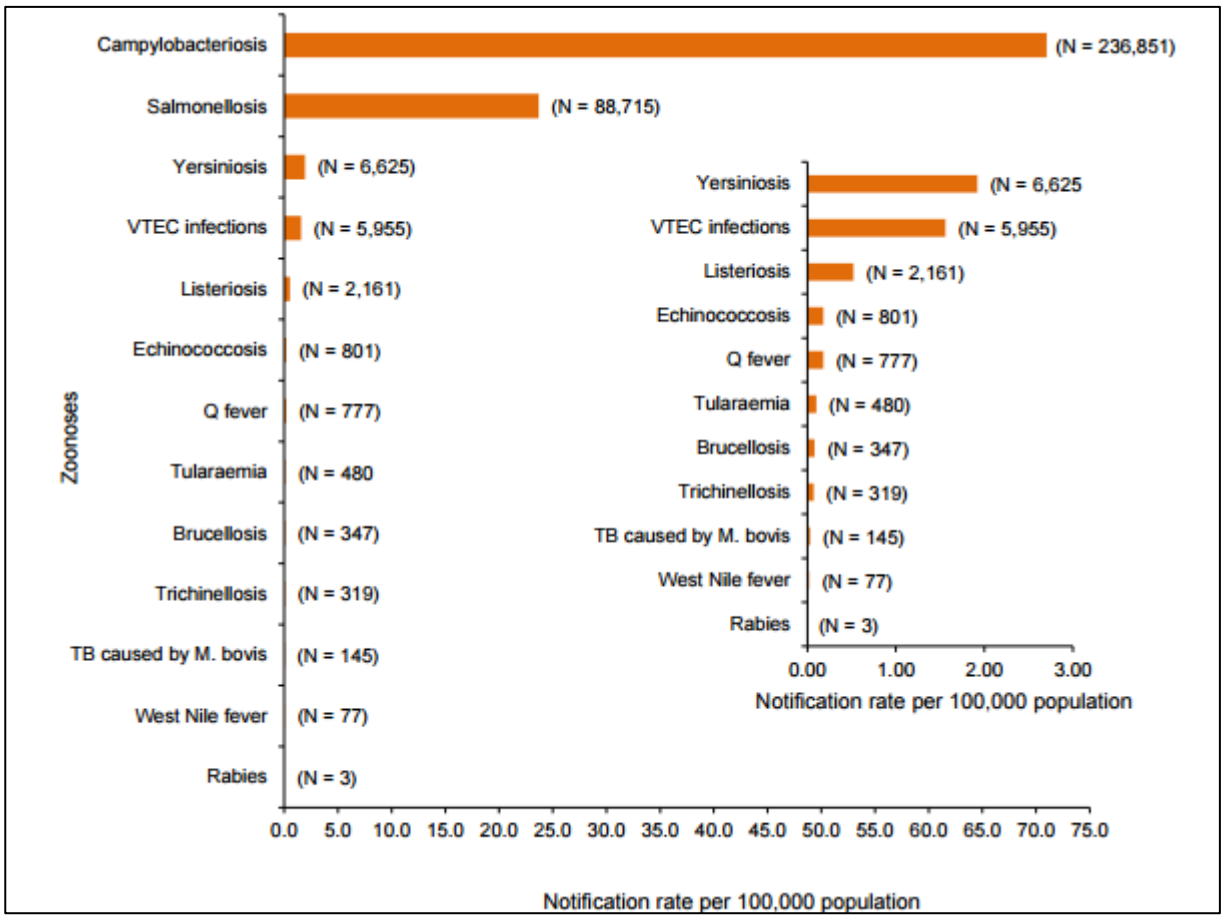


Figure 2. Reported numbers and notification rates of confirmed human zoonoses cases in the EU, 2014 (EFSA and ECDC, 2015)

Country	2014					2013		2012		2011		2010	
	National coverage[a]	Data format[a]	Total cases	Confirmed cases & rates		Confirmed cases &rates		Confirmed cases & rates		Confirmed cases & rates		Confirmed cases &rates	
				Cases	Rate	Cases	Rate	Cases	Rate	Cases	Rate	Cases	Rate
Austria	Y	C	6,514	6,514	76.6	5,731	67.8	4,710	56.0	5,129	61.0	4,404	52.7
Belgium[b]	N	C	8,098	8,098	–	8,148	–	6,607	–	7,716	–	6,047	–
Bulgaria	Y	A	144	144	2.0	124	1.7	97	1.3	73	1.0	6	0.1
Croatia	Y	A	1,647	1,647	38.8	–	–	–	–	–	–	–	–
Cyprus	Y	C	40	40	4.7	56	6.5	68	7.9	62	7.4	55	6.7
Czech Republic	Y	C	20,902	20,750	197.4	18,267	173.7	18,287	174.1	18,743	178.7	21,075	201.5
Denmark	Y	C	3,773	3,773	67.0	3,772	67.3	3,720	66.7	4,060	73.0	4,037	72.9
Estonia	Y	C	308	285	21.7	382	28.9	268	20.2	214	16.1	197	14.8
Finland	Y	C	4.889	4,889	89.7	4,066	74.9	4.251	78.7	4.267	79.4	3,944	73.7
France[c]	N	C	5,958	5,958	45.2	5,198	39.6	5,079	38.9	5,538	42.6	4,324	33.5
Germany	Y	C	70,972	70,530	87.3	63,271	77.3	62,504	76.5	70,812	86.8	65,108	79.6
Greece[d]	–	–	–	–	–	–	–	–	–	–	–	–	–
Hungary	Y	C	8,490	8,444	85.5	7,247	73.5	6,367	64.4	6,121	62.4	7,180	72.9
Ireland	Y	C	2,595	2,593	56.3	2,288	49.8	2,391	52.2	2,433	53.2	1,660	36.5
Italy[b]	N	C	1,252	1,252	–	1,178	–	774	–	468	–	457	–
Latvia	Y	C	38	37	1.8	9	0.4	8	0.4	7	0.3	1	0.0
Lithuania	Y	C	1,184	1,184	40.2	1,139	38.3	917	30.5	1,124	36.8	1,095	34.9
Luxembourg	Y	C	873	873	158.8	675	125.7	581	110.7	704	137.5	600	119.5
Malta	Y	C	288	288	67.7	246	58.4	220	52.7	220	53.0	204	49.3
Netherlands[e]	N	C	4,159	4,159	47.5	3,702	42.4	4,248	48.8	4,408	50.9	4,322	50.1
Poland	Y	C	652	650	1.7	552	1.4	431	1.1	354	0.9	367	1.0
Portugal[d]	–	–	–	–	–	–	–	–	–	–	–	–	–
Romania	Y	C	256	256	1.3	218	1.1	92	0.5	149	0.7	175	0.9
Slovakia	Y	C	6,867	6,744	124.5	5,845	108.0	5,704	105.5	4,565	84.7	4,476	83.0
Slovenia	Y	C	1,184	1,184	57.4	1,027	49.9	983	47.8	998	48.7	1,022	49.9
Spain[f]	N	C	11,481	11,481	82.3	7,064	50.4	5,548	47.4	5,469	46.9	6,340	54.6
Sweden	Y	C	8,288	8,288	85.9	8,114	84.9	7,901	83.3	8,214	87.2	8,001	85.7
United Kingdom	Y	C	66,790	66,790	103.9	66,465	104.0	72,560	114.3	72,150	114.5	70,298	112.5
EU Total	–	–	237,642	236,851	71.0	214,784	64.8	214,316	65.9	223,998	69.0	215,395	67.0
Iceland	Y	C	142	142	43.6	101	31.4	60	18.8	123	38.6	55	17.3
Norway	Y	C	3,386	3,386	66.3	3,291	65.2	2,933	58.8	3,005	61.1	2,682	55.2
Switzerland[g]	Y	C	7,565	7,565	92.9	7,481	93.1	8,432	106.0	7,963	101.2	6,611	84.9

(a): Y: yes; N: no; A: aggregated data; C: case-based data; –: no report.
(b): Sentinel surveillance; no information on estimated coverage thus notification rate cannot be estimated.
(c): Sentinel surveillance; notification rates calculated on estimated coverage of 20%.
(d): No surveillance system.
(e): Sentinel surveillance; notification rates calculated on estimated coverage of 52%.
(f): Sentinel surveillance; notification rates calculated on estimated coverage of 30% in 2013–2014 and 25% in 2009–2012.
(g): Switzerland provided data directly to EFSA. The human data for Switzerland also include the data from Liechtenstein.

Table 1. Reported human cases of Campylobacteriosis and notification rates per 100.000 in the EU/EEA, by country and year, 2010–2014 (EFSA and ECDC, 2015)

Considering the high number of human campylobacteriosis cases, their severity in terms of reported case fatality was low (0.01%) (Tab. 2) (EFSA and ECDC, 2015).

Disease	Number of confirmed[a] human cases	Hospitalisation				Deaths			
		Status available (%)	Number of reporting MS[b]	Reported hospitalised cases	Proportion hospitalised (%)	Outcome available (%)	Number of reporting MS[b]	Reported deaths	Case-fatality (%)
Campylobacteriosis	236,851	25.4	16	18,303	30.4	73.6	15	25	0.01
Salmonellosis	88,715	32.2	14	9,830	34.4	49.6	15	65	0.15
Yersiniosis	6,625	15.2	12	442	44.0	58.3	14	5	0.13
VTEC infections	5,955	39.9	15	930	39.2	58.6	18	7	0.20
Listeriosis	2,161	38.0	16	812	98.9	64.8	20	210	15.0
Echinococcosis	801	24.0	14	122	63.5	24.6	12	1	0.51
Q- fever	777	NA[c]	NA	NA	NA	51.2	11	1	0.26
Brucellosis	347	62.0	9	142	66.1	41.5	10	0	0.00
Tularaemia	480	47.1	8	92	40.7	49.0	9	0	0.00
Trichinellosis	319	74.6	5	150	63.0	74.9	6	2	0.84
West Nile fever[a]	77	66.2	6	48	94.1	66.2	6	7	13.7
Rabies	3	NA	NA	NA	NA	66.6	3	2	100.0

(a): Exception made for West Nile fever where the total number of cases was included.
(b): Not all countries observed cases for all diseases
(c): NA-not applicable as the information is not collected for this disease.

Table 2. Reported hospitalisation and case-fatality rates due to zoonoses in confirmed human cases in the EU, 2014 (EFSA and ECDC, 2015)

Broiler meat is considered to be the most important single source of human campylobacteriosis. In 2014, 38.4% of the 6.703 samples of fresh broiler meat (single or batch, aggregated data from all sampling stages), reported by 18 Member States and sampled at slaughter, processing and retail, were found to be *Campylobacter* positive (Tab. 3). The proportion of positive samples reported in 2014 was comparable to that in 2013, where 31.4% of samples were found to be positive (n = 8,022, 18 Member States). The small increase is most likely a result of varying reporting from the Member States. The proportion of *Campylobacter*-positive samples of broiler meat varied greatly between reporting Member States (Tab. 3). In 2014, *Campylobacter* was detected in 35.5% of single samples at retail with six of eleven Member States reporting at retail level found ≥ 50.0% positive samples. At slaughterhouse level, 44.4% of the single samples tested positive for *Campylobacter*. Reporting data of Spain was at all levels (slaughterhouse, processing plant and retail) and the proportion of *Campylobacter*-positive samples decreased between these stages (EFSA and ECDC, 2015).

Sampling stage	Country	Matrix	Description	Sample origin	Sampling unit	Sample weight	Tested	Positive	Percent positive
	Ireland	fresh	food sample - meat, Surveillance	Germany	single	25 Gram	1	0	0
				Ireland	single	25 Gram	1	0	0
	Netherlands	fresh	food sample	Netherlands	single	25 Gram	589	161	27.33
	Slovakia	fresh, frozen	food sample, Surveillance	European Union	single	25 Gram	9	0	0
	Slovenia	fresh, chilled	food sample, Monitoring		single	1 Gram	50	25	50
	Spain	fresh	food sample	Unknown	single	25 Gram	76	16	21.05
	Sweden	fresh	food sample, Surveillance		single	25 Gram	1	0	0
	Iceland	fresh, frozen	food sample, Surveillance	European Union	single	.	86	2	2.33
Slaughter batch							0	0	.
Batch							51	14	27.45
Single							1,519	558	36.73
Total Retail							1,570	572	36.43
Processing plant	Austria	fresh	food sample, Surveillance	Austria	single	25 Gram	12	5	41.67
	Hungary	fresh, chilled	food sample - meat, Surveillance		single	25 Gram	322	86	26.71
	Poland	fresh	food sample		single	25 Gram	334	2	0.6
			food sample - meat		single	25 Gram	12	0	0
						500 Gram	507	5	0.99
	Portugal	fresh	food sample - meat, Surveillance	Portugal	single	25 Gram	53	22	41.51
	Spain	fresh	food sample	Unknown	single	25 Gram	8	3	37.5
Slaughter batch							0	0	.
Batch							0	0	.
Single							1,248	123	9.86
Total Processing plant							1,248	123	9.86
Slaughterhouse	Austria	fresh	food sample, Surveillance	Austria	single	25 Gram	6	4	66.67
	Belgium	carcase	Surveillance		single	1 Gram	545	119	21.83
	Croatia	carcase	food sample - neck skin, Monitoring	Croatia	single	25 Gram	924	636	68.83
	Cyprus	carcase	animal sample - caecum	Cyprus	single	.	327	195	59.63
	Denmark	fresh, chilled	food sample - meat, Monitoring	Denmark	single	10 Gram	927	238	25.67
	Estonia	carcase	food sample - neck skin, Monitoring	Estonia	batch	25 Gram	12	0	0
	Poland	carcase	food sample - carcase swabs		single	25 Gram	503	253	50.3
			food sample - meat		single	25 Gram	7	0	0
	Spain	carcase	food sample	Unknown	single	25 Gram	131	52	39.69
	United Kingdom	carcase	food sample - neck skin, Survey		slaughter batch	.	497	380	76.46
Slaughter batch							497	380	76.46
Batch							12	0	0
Single							3,370	1,497	44.42
Total Slaughterhouse							3,879	1,877	48.39
Unspecified	Ireland	fresh	food sample - meat, Surveillance	Ireland	single	25 Gram	1	1	100
	Sweden	fresh	food sample, Surveillance		single	25 Gram	3	0	0
		fresh, frozen	food sample, Surveillance		single	25 Gram	2	1	50
Slaughter batch							0	0	.
Batch							0	0	.
Single							6	2	33.33
Total Unspecified							6	2	33.33
Slaughter batch							497	380	76.46
Batch							63	14	22.22
Single							6,143	2,180	35.49
Total (MS)							6,703	2,574	38.4

Table 3. *Campylobacter* in fresh broiler meat, 2014 (EFSA and ECDC, 2015)

Most cases of campylobacteriosis in humans are sporadic and the sources and routes of transmission are more difficult to trace. Although infection can be sporadic, typing systems help to determine the similarity between strains of the same species isolated from human cases, from broiler chickens poultry and from broiler chicken products. Identification of these isolates can help to study the pathogenesis of infections, detect and investigate outbreaks, and assist with surveillance and prevention of campylobacteriosis in humans. The systems typing can be divided into phenotypic and genotypic methods. The first is based on the analysis of the phenotypic characteristics of the microorganisms, as the presence or absence of biological or metabolic activities expressed by the microorganism. The most popularly used phenotypic methods to differentiate *Campylobacter* isolates include biotyping, serotyping, and multilocus enzyme electrophoresis. The genotypic methods, instead, provide to examine whole genome, allowing for the differentiation of many subtypes within a species as well as the genomic relatedness among isolates (Eberle and Kiess, 2012).

1.1.3.1 Phenotypic Methods

The techniques for differentiating isolates phenotypically are based on the presence or absence of biological or metabolic activities expressed by the microrganism. The most popularly phenotypic methods to differentiate *Campylobacter* isolates include biotyping, serotyping, and multilocus enzyme electrophoresis (Eberle and Kiess, 2012).

Biotyping differentiate the bacterial isolates through the expression of metabolic activities that allow the survival, growth, and development of microorganisms in certain substrate. These metabolic activities include colonial morphology, such as size, shape, staining characteristics (Gram stain), sporulation characteristics, mechanisms of motility, biochemical and physiological characteristics and fatty acid methyl ester composition (FAME) (Bisen *et al*., 2012; Eberle and Kiess, 2012).

Serotyping is based on the knowledge that microorganisms have differing cellular surface structures; this method use specific antibodies and antisera to detect different surface structures of bacteria as antigens. *Campylobacter* spp. have several structures found on their cell surface, including lipopolysaccharides, capsular polysaccharides, membrane proteins, and extracellular organelles; most of these structures also have a role in host-bacterium interactions (Eberle and Kiess, 2012).

The Penner scheme, developed by John Penner of the University of Toronto, was based on a passive hemagglutination method using soluble antigen extracts of isolates and specific antisera raised on the antigens of *Campylobacter*. This technique differentiates *Campylobacter* strains on the basis of heat-stable (HS) antigens found on the cell. The Lior scheme, developed by Hermy Lior at the National Laboratory of Enteric Pathogens (NLEP) located in Ottawa, was based on a slide

agglutination procedure using live bacteria together with unabsorbed and absorbed antisera. This technique detects heat labile (HL) antigens. Recently, a modification of the Penner serotyping procedure was developed at the Laboratory for Enteric Pathogens, Central Public Health Laboratory, located in London. This serotyping assay was based on the same principle as that developed by Penner and also detected HS antigens of *Campylobacter*, though it did not use a passive hemagglutination procedure; rather, antigens were detected by direct bacterial agglutination of heated suspensions by using specific antisera in microtiter plates (Woodward and Rodgers, 2002).

Multilocus Enzyme Electrophoresis

Multilocus enzyme electrophoresis (MEE) is based on the relative electrophoretic mobilities of intracellular enzymes of bacterial isolates by generating an electrophoresis under non denaturing conditions. The variation of the electrophoretic mobility of an enzyme is dependent on mutations at the gene locus that causes amino acid substitutions, which alter the charge of the protein. These mobility variants are called electromorphs. The unique profile of an electromorph produced by each strain of a bacterial isolate is called an electromorph type (ET) (Eberle and Kiess, 2012).

1.1.3.2 Genotypic Methods

Genotype methods have the advantage to allow the investigation of foodborne outbreaks, give better understanding into the epidemiology of infections and aid in the treatment of infested people (Adzitey *et al*., 2013).

Indeed, these methods help to understand if the isolates, recovered from a localized outbreak of disease, are the same or are different strains; furthermore these technique are appropriate to investigate if the strains that cause disease in one geographic area are related to those isolated world-wide (Maiden *et al*., 1998).

This techniques provide more sensitive strain differentiation and higher levels of standardization, reproducibility, versatility and discriminatory power than phenotypic typing methods. The isolates can be assigned as belonging to a "type" by a typing technique (Adzitey *et al*., 2013). The common genotypic typing methods include Single-enzyme Amplified fragment-length polymorphism (s-AFLP), Multilocus sequence typing (MLST), Pulsed field gel electrophoresis (PFGE), flaA Short variable region (flaA-SVR) (Clark *et al*., 2012).

Single-enzyme Amplified fragment-length polymorphism (s-AFLP)

The method was originally developed for the typing of crop plants and is a high-resolution genotyping method that has been widely applied to both eukaryotic and prokaryotic organisms (Vuylsteke *et al*., 2007). The AFLP procedure provides the digestion of genomic DNA with a single enzyme; the ligation of an adapter, designed to disrupt the enzyme restriction site, to each sticky end of the digestion fragments; PCR amplification of the adapter-tagged fragments with a single primer which is complementary to the adapter sequence and electrophoresis separation and detection with ethidium bromide of the amplified fragments in a agarose gel. The power of AFLP analysis derives from its ability to quickly generate large numbers of marker fragments for any organism, without prior knowledge of genomic sequence (Gibson *et al*., 1998).

Pulsed field gel electrophoresis (PFGE)

PFGE is considered as the 'gold standard' typing method and enabled to perform epidemiological relationships between isolates and is the most discriminatory molecular methods for molecular epidemiological studies and for typing *Campylobacter* spp. This method has good reproducibility, discriminatory power and typeability but PFGE is sensitive to genetic instability.

Pulsed field gel electrophoresis (PFGE) is an agarose gel electrophoresis technique that provides the separation of large DNA molecules in an agarose gel matrix by applying an electric field that

periodically changes direction (three directions) in a gel matrix unlike the conventional gel electrophoresis where the current flows only in one direction (Adzitey *et al*., 2013).

This method has been widely used for characterizate foodborne pathogens and was the basis for the implementation of PulseNet in the USA to aid in the rapid detection of local and multistate outbreaks (Boxrud *et al*., 2010). This technique begins by embedding bacterial cells in agarose gel, referred to as the plugs. The plugs are then treated with enzyme and RNases to digest unwanted protein and RNA, leaving purified chromosomal DNA. Then, the plugs are cut into segments and treated with restriction enzymes, typically with the enzymes *KpnI* or *SmaI*, to digest the DNA into a small number of large fragments that are then separated electrophoretically based on size (Eberle and Kiess, 2012).

Multilocus sequence typing (MLST)

Multilocus sequence typing (MLST) has been successful in the characterization of several other bacteria. This technique employs the same philosophy as multilocus enzyme electrophoresis, in that neutral genetic variation from multiple chromosomal locations is indexed, but exploits nucleotide sequence determination to identify this variation (Dingle *et al*., 2001). The scheme of tecnique requires the sequencing of short DNA fragments within seven housekeeping genes. Sequenced PCR products for each of the loci (*asp*, *glnA*, *gltA, glyA*, *pgm*, *uncA*, *tkt*) are assigned an allele number based on a complete match to an allele in the global PubMLST database. The array of seven allele numbers is then assigned a sequence type (ST) according to the database. Sequence types that share four or more alleles belong to the same clonal complex (CC) or lineage (Taboada *et al*., 2013).

flaA Short variable region (flaA-SVR)

The flagellin gene locus of *C.jejuni* contains two flagellin genes (*fla*A and *fla*B), which are arranged in tandem and are separated by approximately 170 nucleotides. The flagellin (*flaA*) gene has provided a useful target for discriminating among *C. jejuni* isolates. Subtyping methods based on

*fla*A typing include *fla*A restriction fragment length polymorphism analysis (*fla*A-RFLP), and sequencing of a 321 bp short variable region of the *fla*A gene (*fla*A-SVR) (Corcoran *et al*., 2006).

1.2 *Campylobacter* infections

1.2.1 Sources of infections

1.2.1.1 *Campylobacter spp* in broiler chickens products

The chicken products are considered the principle vehicles of transmission of campylobacteriosis in human populations worldwide. *Campylobacter* spp can colonize the intestinal mucosa of most warm-blooded hosts as cattles, sheeps, swines, layers, turkeys, ducks and avian species. The avian species are the most common hosts for *Campylobacter* spp because of their higher body temperature (Sahin *et al*., 2015).

Therefore, the preparation, consumption and handling of raw or undercooked broiler chicken is recognized as a significant source of food-related transmission *Campylobacter* species to humans, particularly in summer (Silva *et al*., 2011). There are many points in processing of poultry at which carcasses and products can become newly contaminated o cross-contaminated with microorganisms. In Europe the prevalence of *Campylobacter* in retail raw poultry products was 53,3%; however, prevalence between European countries ranges from 8.1 to 80.0% (Suzuki and Yamamoto, 2009).

1.2.1.2 *Campylobacter spp* in other foods and in other sources

The consumption of unpasteurized or inadequately pasteurized cow's milk (Schildt *et al*., 2006) and of contaminated drinking water (Silva *et al*., 2011) are potential sources of campylobacteriosis in humans. Milk and products derived from milk of dairy cows are considered as important sources of foodborne pathogens to humans. In Italy, contamination of milk by *C. jejuni* has assumed more importance since 2004, after the enactment of European Commission Regulation 853/2004, when the sale of raw milk for human consumption by self-service vending machines has been allowed. Two outbreaks in the Emilia Romagna Region in 2008 and 2009, one in the Veneto Region and another in the Marche Region were reported following raw milk consumption. In 2011, the Italian Ministry of Health reported a national prevalence of 2.3% for thermotolerant *Campylobacter* spp. in raw milk sampled from automatic vending machines (Giacometti *et al*., 2013; Bianchini *et al*., 2014). The presence of *Campylobacter* in milk is due to direct contact with contaminated sources in the dairy farm environment, to contamination with the faeces of *Campylobacter* infected cows, and to excretion from the udder of an infected animal (Oliver *et al*., 2005; Modi *et al*., 2015).

Also untreated water is an effective vehicle of transmission of *Campylobacter* species to humans (Denis *et al*., 2011). The drinking water can be fecally contaminated either by runoff of surface water after rain or by leakage of a sewage pipe close to the drinking water pipeline (Hänninen *et al*., 2003).

Furthermore, some studies indicated that ready to eat (RTE) foods, such as meat products, salad vegetables and raw milk cheeses, are a risk factor for *Campylobacter* infection (De Jong *et al*., 2008; Medeiros *et al*., 2008).

The association with ready to eat foods and salad may be explained by cross-contamination due incorrect handling of food within the kitchen (Chai *et al*., 2008b).

Also, the contact with animals is associated with *Campylobacter* infection. Low frequencies of human-to-human transmission has also been observed (Wieczorek and Osek, 2013).

1.2.2 Clinical manifestation in humans

In developing countries, campylobacteriosis is the most common human gastroenteric infection. The two predominant species causing gastrointestinal infections are *Campylobacter jejuni* (80%) and *Campylobacter coli* (10%); however, other species such as *Campylobacter lari*, *Campylobacter upsaliensis* and *Campylobacter fetus* have also been associated with gastrointestinal disease in humans (Skarp *et al*., 2016).

The infectious dose is not exactly determined, but disease has been experimentally induced with as few as 500 bacterial cells in adult volunteers. The infective dose depends upon a number of factors including the virulence of the strain, the vehicle in which it is ingested and the susceptibility of the individuals (Wieczorek and Osek, 2013). Young children (1-4 years of age), very old patients (>75 years of age), pregnant women and immunocompromised adults appear particulary susceptibility to *Campylobacter* infection.

Human campylobacteriosis typically develops 1–5 days after exposure and the infection results in an acute, self-limited gastrointestinal illness characterized by fever, watery and sometimes bloody diarrhoea, abdominal cramps and vomiting lasting for approximately 5–7 days (Skarp *et al*., 2016).

Clinically, *Campylobacter* infection is indistinguishable from acute gastrointestinal infections produced by other bacterial pathogens, such as *Salmonella, Shigella*, and *Yersinia* species (Allos, 2001). *Campylobacter* species have been associated with a range of gastrointestinal diseases, including inflammatory bowel diseases (IBD) and periodontitis. Extra-gastrointestinal manifestations of *Campylobacter* infection are quite rare and may include bacteremia, brain abscesses, meningitis, and reactive arthritis (Kaakoush *et al*., 2015).

The most important severe post-infectious complications are Guillan-Barré syndrome (GBS) and Miller Fisher syndrome, that are acute immune-mediated neuropathies.

GBS is an immune-mediated demyelinating polyneuropathy of peripheral nervous system (PNS) characterized by acute or subacute symmetrical ascending motor weakness, areflexia, and mild to moderate sensory abnormalities.

GBS that occurs 1 to 3 weeks after *C. jejuni* infection has become the most common cause of acute flaccid paralysis with an annual incidence of 0.6-4 cases per 100.000 populations (Nyati and Nyati, 2013). The clinical features of GBS were described by Landry in 1859. In 1916, Guillain Barré, and Strohl found an increase of protein concentration and normal cell count in cerebrospinal fluid (CSF) of two French soldiers (Burns, 2008). GBS has been estimated to occur about once in every 1000 cases of campylobacteriosis (Allos, 1997). It has been shown that GBS arises as a result of autoimmune attack due to molecular mimicry that exists between certain lipopolysaccharide (LPS) molecules of *C. jejuni* strains and human nerve tissue gangliosides. Furthermore, some serotypes of *C. jejuni* are associated with GBS (Yu *et al*., 2006). This disease is characterized by a progressive symmetrical weakness in the limbs, with or without hyporeflexia, which can also affect respiratory and cranial nerve-innervated muscles. Many patients have pain and fatigue that can persist for months or years while 3–10% of patients die and 20% are still unable to walk after 6 months (Van Doorn *et al*., 2008).

Miller Fisher syndrome is a clinical variant of GBS that was discovered in 1956 by Charles Miller Fisher. It is characterized by the acute development of ataxia (eye muscle weakness),

ophthalmoparesis (absence of reflexes), and areflexia (inability to coordinate voluntary muscular movements such as walking) and, in some cases, facial and bulbar palsy. Miller Fisher's syndrome generally is self-limited and has an excellent prognosis (Teener, 2012).

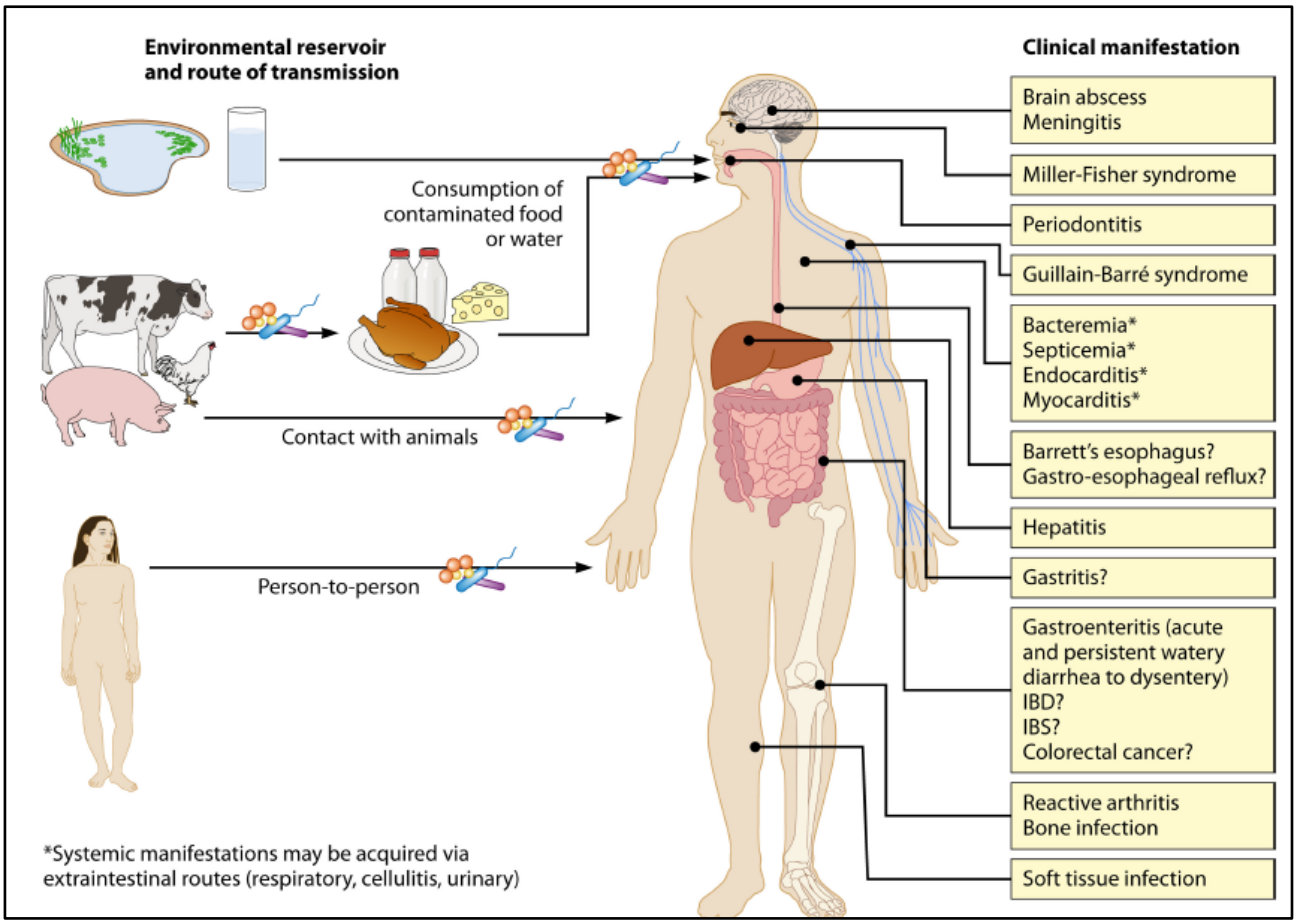


Figure 3. Sources of infection and clinical manifestations associated with *Campylobacter* species. Abbreviations: IBD, infiammatory bowel diseases; IBS, irritable bowel syndrome. Question marks indicate conditions for which a role for *Campylobacter* is implicated but not certain (Kaakoush *et al*, 2015)

1.2.3 Therapy and antibiotic resistance

Most *Campylobacter* infections are self-limiting and require no antimicrobial treatments other than supportive treatment, such as maintenance of proper hydration and electrolyte balance (Lagler *et al*., 2016). However, antibiotics are needed only in patients who are immunologically compromised, in patients with severe or persistent symptoms, and those with extraintestinal infections (Kaakoush *et al*., 2015). Macrolides, such as erythromycin, are the preferred choice of therapy in the treatment of *Campylobacter* infections, following from fluoroquinoles, such as ciprofloxacin; also tetracyclines are used clinically for the treatment of infectious caused by *Campylobacter* spp. Since the late 1980s, however, there has been an increase of resistance to macrolides and fluoroquinoles and the treatment of this disease has become complicated (Wieczorek and Osek, 2013). Quinolones and floroquinolones are an important class of wide spectrum synthetic antibacterial agents against enteric pathogens (Prabodh *et al*., 2009). The first quinolone, nalidixix acid, was introduced in 1962, and showed a narrow spectrum of activity. The evolution of quinolones to more potent molecules was based on changes at positions 1, 6, 7 and 8 of the chemical structure of acid nalidixic (Fàbrega *et al*., 2009). Quinolones inhibit the synthesis of bacterial DNA causing cell death. The primary target of quinolone antibiotics are DNA gyrase and topoisomerase IV, which are enzymes essential for DNA replication, transcription, recombination, and repair. Quinolone resistances arises as a result of alterations in DNA gyrase and topoisomerase IV, as well as through changes in drug entry and efflux. In *Campylobacter* species, fluoroquinolone

resistance mechanisms appear to be mainly due to mutations in the *gyrA* gene encoding part of the GyrA subunit of DNA gyrase. The Thr-86-Ile change, mediated by the C257T mutation in the quinolone-determining region (QRDR) of the gyrA gene, is the most frequently observed mutation in fluoroquinolones resistant isolates of *Campylobacter* and confers high level of resistance to this group of antimicrobials (Wieczorek and Osek, 2013). The genus encoding topoisomerase IV (*parC/parE*) are also involved in fluoroquinolones resistance in Gram-negative bacteria; however, these genes are no present in *Campylobacter* (Han *et al*., 2012).

Macrolides are mostly produced by *Streptomyces* and related bacteria. Erythromycin is produced as a natural product by the bacterium *Saccharopolyspora erythraea* and it is the first macrolide antimicrobial introduced in 1952 (Roberts *et al*, 1999). The macrolides are widely used antimicrobial agents and considered to be safe and effective drugs. Their antimicrobial spectrum covers most of Gram-positive and the Gram-negative microorganisms, including *Campylobacter* .

Macrolides interrupt protein synthesis in bacterial ribosomes by binding to the 50S subunits and interfere with protein synthesis by inhibiting the elongation of peptide chains. Studies in *C. jejuni* and *C.coli* demonstrated that the resistance to macrolides was associated with nucleotide mutations at positions 2058 and 2059 in the peptidyl transferase region in domain V of the 23S rRNA, the target of macrolides. Therefore, some studies have shown that macrolide resistance in *C.jejuni* and *C.coli* isolates may also involve an efflux pump, which contributes to the multidrug resistance in clinical bacteria (Harrow *et al*., 2004; Gibreel *et al*., 2005).

Tetracyclines (Tc), discovered in the 1940s, are a broad-spectrum antibiotics that exbihited activity against a wide range of microorganisms, both Gram-positive and Gram-negative bacteria, and have been used extensively in a variety of medical and veterinary infections (Chopra and Roberts, 2001).

In *C. jejuni* and *C. coli* the resistance to tetracyclines is conferred by the *tet(O)* gene carried on transmissible plasmids; *tet*(O) has also been found to be chromosomally located. The Tet(O) protein belongs to the class of ribosomial protection protein that confer tetracycline resistance.

Another problem related to antibiotic resistance in *Campylobacter* infections is the emergence of multidrug-resistant (MDR) strains, defined as strains with resistance to three or more antibiotics, which have been isolated in many countries throughout the world.

The potential risk that macrolide-fluoroquinole-tetraciclyne resistance *Campylobacter* spp will be transmitted from broiler chickens products to humans has raised concerns that using these antimicrobials in broiler chickens will compromise the treatment of human infections (Kaakoush *et al*, 2015).

1.3 Pathogenesis of *Campylobacter* infection

1.3.1 Virulence factors of *Campylobacter spp*

Specific virulence mechanisms have not yet been clearly elucidated for *Campylobacter* spp probably due to the lack of pathogenesis similarity between *Campylobacters* and other pathogens (Silva *et al*., 2011). Virulence factors include flagella mediated motility, bacterial adherence to intestinal mucosa, invasive - translocation capabilities and the ability to produce toxins and secreted proteins (Biswas *et al*., 2011).

The polar flagella of *Campylobacter* microorganisms imparts an unusual rapid, darting motility, which is characteristic of those pathogens; these structures have long been recognized as crucial to pathogenesis. This motility is absolutely required for colonization of gastrointestinal tract of human (Guerry, 2007). Invasion of the colonic mucosa, which causes cellular inflammation, is probably resulting from the production of cytotoxins, and is followed by the reduction of the absorptive capacity of the intestine (Silva et al., 2011). In vitro, a stress response, resulting in increased regulation of molecules involved in glycolysis, anti-apoptotic mechanisms, oxidative stress and

inflammatory kinase, was observed in the human colonic enterocyte like Caco-2 cells, previously infected with clinical isolates of *C.jejuni* (Ketley and Konkel, 2005).

It is thought that the capacity of this pathogen to reach the intestinal tract is, in part, due to resistance to gastric acids and also to bile salts, even though the disease severity may depend on the virulence of the strain as well as on the host's immune condition (Silva *et al*., 2011).

1.3.1.1 Flagella

Flagella were recognized to be important for pathogenesis and their role under different chemotactic conditions is essential for bacterial survival in the various ecological niches encountered in the gastrointestinal tract (Silva *et al*., 2011). The flagella are composed of a major flagellin, FlaA, and a minor flagellin, FlaB, that are both predicted to be approximately 59 kDa in size and displays 98% homology. In *Campylobacter jejuni* and *C.coli*, two genes, *fla*A and *fla*B regulated by the classic flagellin promoter σ28 and by dependent promoter σ54, respectively, are involved in expression of the flagellar filament (Guerry, 2007).

Mutation of *flaA* gene ($flaA^{-}B^{+}$) in *C. jejuni* produces a severely truncated flagellar filament, and these bacteria are non-motile. In contrast, mutation of gene *flaB* ($flaA^{+}B^{-}$) in *C. jejuni*, have no significant change in motility and these bacteria are motile (Neal-McKinney *et al*., 2010). The presence of both gene products in the filament is required for maximum motility, though FlaA is the predominant constituent of the flagellar filament (Alm *et al*., 1993).

Interestingly, several studies have indicated that flagella expression is required to biofilm formation, which is in contrast to biofilm formation for most bacteria. Different study revealed that *C. jejuni* can form biofilms on inert surfaces and this ability may help explain its resistance to survive outside its normal host such as in water and in other harsh environmental conditions constituting a source of contamination for animals and human (Teh *et al*., 2014; Guerry, 2007; Reeser *et al*., 2007)

1.3.1.2 Cytolethal Distending Toxin (CDT)

The cytolethal distending toxin (CDT) is one of the main virulence factors which cause pathogenesis in humans and animals, interfering with the cell division and differentiation of cells in intestinal crypts (De Carvalho *et al*., 2013). The result of CDT activity can differ depending on the type of eukaryotic cell affected. CDT contributes to campylobacteriosis by inhibiting both cellular and humoral immunity via apoptosis of immune cells and also by generating necrosis of epithelial cells and fibroblasts involved in the repair of lesions produced by pathogens (Smith and Bayles, 2006).

CDT is produced by a variety of Gram-negative bacteria including *Campylobacter* spp., *Escherichia coli*, *Shigella* spp., *Helicobacter* spp., *Haemophilus ducreyi* and *Actinobacillus actinomycetemcomitans.* CDT activity is encoded by the *cdt* gene operon, made up of three adjacent genes, termed *cdtA, cdtB* and *cdtC*. The presence and expression of the three genes are required for its functional activity, with CdtB subunit responsible for toxin activity. CdtA and CdtC subunits are, instead, responsible for binding to the susceptibility cells. It has been suggested that the *cdt* genes may be universally present in *C. jejuni* and *C. coli* and have a distinct species divergence (Asakura *et al*., 2008).

CDT attaches itself to the human cell membrane and the binding is dependent on the presence of intact lipid rafts. The toxin is internalized via dynamin-dependent endocytosis into early and late endosomes. The CdtB subunit further transits to the Golgi complex, and is then retrogradely transported to the endoplasmic reticulum (ER). Translocation from the ER does not require the ER-associated degradation (ERAD) pathway, and protein unfolding (Fig. 4) (Guerra *et al*., 2011).

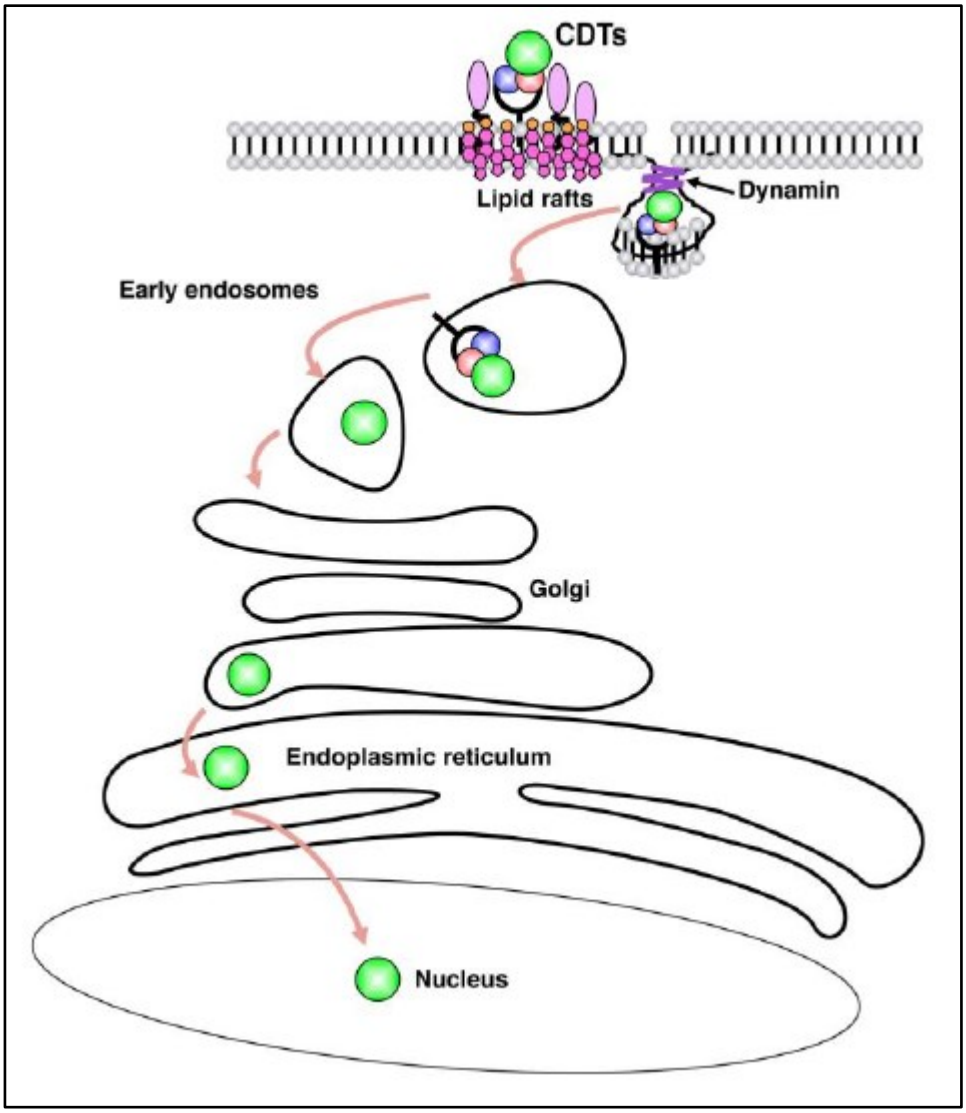


Figure 4. International pathway of Cytolethal distending toxin (CDT) (Guerra *et al*., 2011)

1.4 *Campylobacter* in the broiler chickens production chain

Theoretically, high level of biosecurity, including improvement in personnel hygiene practices, on the poultry farm should be the primary strategy to prevent *Campylobacter* introduction into broiler flocks (Wagenaar *et al*., 2013). Reducing the prevalence of *Campylobacter* colonization in broiler chickens on farms will decrease the introduction of high numbers of *Campylobacter* into the slaughterhouse. This may result in a low concentration or absence of *Campylobacter* on the final product (Wagenaar *et al*., 2006). A high level of biosecurity on farm level should prevent the introduction of *Campylobacter* into a flock but it does not guarantee a *Campylobacter*-free flock at slaughter (Wagenaar *et al*., 2013). Furthermore, good hygienic practices should be applied during transportation, in slaugherhouse, during the processing and the packaging of final broiler chicken products, regarding major points of cross-contamination on the line. In addition, good hygiene practices of retailers in the private kitchen and in restaurants, are needed to avoid the cross-contamination and to reduce the risk of human infections (EFSA, 2011b; De Jong *et al*., 2008).

1.4.1 *Campylobacter* in broiler chickens farms

Flocks at commercial production systems consist of approximately 10.000–30.000 birds per house (Skarp *et al*., 2016).

Campylobacter-positive broiler chickens, in general, can establish persistent and benign infections in broiler chickens. In most flocks, colonization is not detectable until at least 10 days of age (the so-called lag phase of infection) regardless of production types (both conventional and free-range or organic), indicating that the naturally acquired flock colonization is age dependent (Newell and Fearnley, 2003).

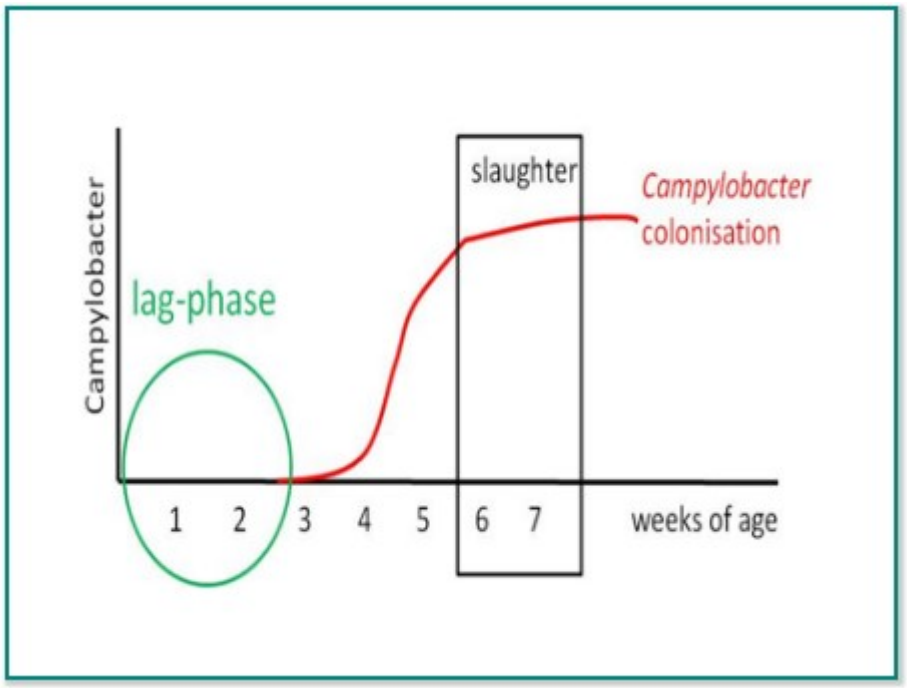


Figure 5. Susceptibility to broiler chickens to *Campylobacter* spp, Institute of Infection and Global Health, University of Liverpool

During their first week of life, the birds showed an apparent resistance to *Campylobacter* infection. The reasons for this lag phase are unknown but might be attributed to multiple factors, such as presence of maternal antibodies in broiler chicks, antibiotic feed additives, intestinal development, and intestinal microbiota (Lin, 2009). As chickens are coprophagic, the fecal–oral spread of

Campylobacters is an important factor in the dissemination of microorganisms around broiler flocks; however, the rapidity of the shift from uncolonized to 100% colonized in only few days suggests that *Campylobacters* can also spread from broiler chicken to broiler chicken via their communal source of drinking water. In this way, the overall prevalence within the flock reaches the highest level (close to 100% in many cases) at the slaughter age (Keener *et al*., 2004). Although young birds may develop clinical disease (e.g., diarrhea and weight loss), the vast majority of studies pointed out the commensal nature of the microorganism in broiler chickens with no clinical signs of disease. In the sporadic events where signs of disease were observed in experimentally *Campylobacter* infected chicks, gross and microscopic lesions were mostly minimal and mainly confined to the gastrointestinal tract. Blood and mucus in the lumen of small intestine can be seen occasionally (Swayne *et al*., 2013). A study revealed that *Campylobacter* may play a role in the pathogenesis of lesions; *Campylobacter* was isolated more frequently from broiler chickens liver with necrotic lesions than from normal livers (Boukraa *et al*., 1991). However, there was no directly evidence that *Campylobacter* contributed to the lesions. As enteric microorganism, *Campylobacters* spp are able to survive the acidic environment of proventriculus and ventriculus as well as in the small intestine, and in the lower intestine, where they establish colonization in cecal and cloacal crypts. The colonized birds can excrete high numbers of the microorganisms from about 10^5 to 10^8 CFU/g of faeces. To a lesser extent, *Campylobacter* can find in the small intestines, liver, and other organs.

From the EU baseline survey (EFSA, 2010b), the season represents an important risk factor during the period July-September compared to January-March for the prevalence of *Campylobacter* positive poultry flocks. Indeed, in several northern European countries, as Sweden, Denmark, Norway and Netherlands, the seasonal variation in *Campylobacter* prevalence in broilers, with a peak in the summer, has been observed.

The reason for the association of higher *Campylobacter* prevalence and season is not known, but

appears to be the warm temperature, found in the summer, that can foster the presence of important vectors, as the flies (EFSA, 2011b).

The prevalence of flock positivity is also dependent on flock size and the type of production system. Flock positivity is generally higher (up to 100%) in organic and free-range flocks compared to intensively reared flocks (Newell and Fearnley, 2003). This can be explained by level of environmental exposure; indeed, while in the conventional and extensive indoor broiler flocks the birds are reared in a confined environment, in organic broiler flocks they easily can close contact to wild birds and theirs faeces as well as to soil and rain water (Heuer, *et al*. 2001; Ahmed *et al*., 2016). Indeed, the presence of *Campylobacter* in water in the open is a possible explanation for the high *Campylobacter* incidence observed in organic broiler (Heuer et al., 2001). Moreover, free range birds, unlike housed bird, can come more easily in contact with infectious agents transmitted by air (Ahmed *et al*., 2016). Several studies (Evans and Sayers, 2000; Hald *et al*., 2001; El-Shibiny *et al*., 2005, Van Overbeke *et al*., 2006) documented a high prevalence of *Campylobacter* in poultry reared under free-range conditions showing that open environment exposure has to be considered as additional risk factor.

The broiler chicken flocks can be colonized by single or multiple species and genotypes of *Campylobacter*, even during a single rearing cycle. In broiler chickens, *C. jejuni* is the predominant species that colonize the flocks, followed by *C. coli* and rarely other species; however, in commercial turkeys and in organic and free-range chickens, *C. coli* has been reported to be the dominant species (Sahin *et al*., 2015).

While the vertical transmission of *Campylobacters* to flocks via contaminated eggs remains controversial, horizontal transmission is considered the most probable mechanism of flock colonization by *Campylobacter* (Newel and Fearnley, 2003).

The lack of a hygiene barrier, the presence of other animals (dogs/cats and wildlife) in proximity to poultry houses, increasing bird slaughter age (from 36 days to > 40 days), size of flocks, the practice of partial depopulation of broiler chickens from the flock during rearing (thinning),

presence of rodents and insects, dirty footwear, inappropriate cleaning of the broiler house can increase the risk of colonization and dissemination of *Campylobacter* in broiler flocks. Furthermore, the presence of contaminated water along the lines and reservoirs of broiler houses and the presence of windows with canvas blinds represented a risk factor for *Campylobacter* infection compared with other kinds of blinds (made mainly of polyethylene). Canvas blinds are usually folded during the cleaning and disinfection of poultry houses; thus, *Campylobacter* could stay in these blinds as a source of infection for the next flock; canvas blinds could also facilitate the access of flies or other vectors to the house (Torralbo *et al*., 2015).

The feed, feed additives, and fresh litter are not potential sources of infection because the dry conditions of feed and fresh litter are considered lethal to *C. jejuni* (Newel and Fearnley, 2003).

1.4.2 *Campylobacter* at slaughterhouse

1.4.2.1 *Campylobacter* and transport of broiler chickens from farm to slaugherhouse

Caging and transport can influence and increase the level of contamination of *Campylobacter* (Northcutt and Berrang, 2006). The stress caused to broiler chickens by transport cages to slaughterhouse increased the rate of fecal shedding, with changes in the consistency of the faeces, to a more liquid nature. This can cause fecal contamination on cage flooring and *Campylobacter* can be transferred to the broiler feathers and skin (Franz *et al*., 2012). Therefore, negative flocks can become externally contaminated during transport, but it is unlikely that broiler chickens of these flocks will be intestinal colonised during transport, unless the transport time is lengthy.

However, this contamination is only of interest if there are birds present that are excreting *Campylobacters* (Klein *et al*., 2007).

The inadequate cleaning and disinfection of transport cages may also introduce *Campylobacter* into the free flocks (Northcutt and Berrang, 2006).

1.4.2.2 Cross-contamination during slaugherhouse and processing

In a commercial poultry slaughter line, up to 13.000 animals per hour are processed. The process is almost completely automated, providing a challenge for hygienic slaughter and carcass preparation (Wagenaar *et al*., 2013). At the processing plant, broiler chicken carcasses pass through several stages before reaching the final product (Guerin *et al*., 2010). After slaughter and blood draining, the broiler chickens pass through a warm water reservoir (50-60°C) to facilitate easy feather removal (this stage is called scalding), followed by de-feathering, evisceration, washing, and air chilling (Guerin *et al*., 2010; Zhuang *et al*., 2013). The scalding process is considered to be an important site of cross-contamination between broiler chicken flocks (Keener *et al*., 2004). *Campylobacter* has been periodically recovered from scald water (Ivanova *et al*., 2014); however, the survival of *Campylobacter* in scald tank is low. The scalding process allows the opening of feather follicles to aid feather removal, and the follicles might remain open throughout processing until the carcass is chilling. When the follicles close during chilling, the microorganisms may be retained (Keener *et al*., 2004). During the plucking and the evisceration process broiler chicken carcasses can become contaminated with fecal material.

The rubber fingers applied in the de-feathering process exert pressure on the carcasses, forcing potential contaminated colon contents out of the vent and spreading it on the carcasses and the slaughter equipment (Rasschaert *et al*., 2006).

Moreover, during evisceration process the intestinal tract may leak or rupture and its contents would be transferred to the skin of the carcass.

Campylobacter spp. can remain entrapped with a surface water layer in skin crevices or feather follicles which provide a favorable environment for survival and cross-contamination. Because *Campylobacter* requests microaerophilic conditions, the microenvironment of the skin enables the persistence and survival of *Campylobacter* in feather follicles at room temperature, 4°C or frozen condition (Silva *et al*., 2011).

Carcasses are commonly washed with potable water to remove contamination, such as blood, tissue fragments, and fecal contamination as part of the regular processing procedures (Keener *et al*., 2004).

The chilling step is a critical control point to reduce carcass contamination and to prevent bacterial growth (Osiriphun *et al*., 2011).

1.4.3 Cross-contamination in domestic kitchen

The cross-contamination of *Campylobacter* during food preparation in kitchen between raw and cooked products, ready to eat foods, salad vegetables via hands, cutting boards, knives or other unwashed surfaces represents a higher risk for consumers than consumption of undercooked or improperly cooked broiler chicken meats (Usha *et al*., 2010). Hands and utensils that have been involved in the preparation of raw broiler should be washed properly and cleaned with natural compounds, as monoglycerides, respectively before further preparation of ready-to-eat foods like fruits and vegetables. Alternatively, separate utensils for preparing raw and ready-to-eat foods must be used. In this way, the final consumer risks can be reduced by preventing cross-contamination and heating foods at adequate temperatures (Thormar and Hilmarsson, 2010).

1.4.4 *Campylobacter* control along the broiler chickens chain

The control of *Campylobacter* along the broiler chicken chain is most effective when the colonization of birds at the farm level can be prevented. This may result in a low concentration or absence of *Campylobacter* on the final product. Reducing the prevalence of *Campylobacter* infection in broiler chicken farms with control measures decreases the contamination of *Campylobacter* in the following steps. The primary strategy for reducing *Campylobacter* infection in broiler chickens is the reduction of environmental exposure (biosecurity measures). Flies can be carriers of *Campylobacter*, and the fly traffic in and out of broiler houses is huge (>30.000 flies per production cycle). Installation of fly screens around ventilation openings showed delayed and reduced *Campylobacter* colonization in flocks (Wagenaar *et al*., 2013).

Vaccination of broiler chickens would be an alternative to administering antibiotics to reduce and prevent colonization of *Campylobacter* in the native host (Neal-McKinney *et al*., 2014). Indeed, it has been observed a correlation between increasing levels of *Campylobacter* antibodies and reducing levels of *C.jejuni* colonization in poultry (Lin, 2009). Actually no vaccine is completely able to prevent or reduce *Campylobacter* colonization in broiler chickens (Wagenaar *et al*., 2013). Moreover, the use of antimicrobial compounds alternatives, such as bacteriophage therapy and bacteriocin treatment, to reduce the numbers of *Campylobacters* at farm level is also a potentially useful intervention strategy (Lin, 2009). Bacteriophages are ubiquitous in the environment and are viruses that can infect and kill susceptible bacteria. Most bacteria, include *Campylobacter*, have their own specific bacteriophages. Like other viruses, they depend on the metabolism of their host cell, their narrow host range is restricted to one bacterial strain or species. Bacteriophages recognized specific receptors on the outer surface of bacteria and inject their DNA into the host bacterium (Clokie *et al*., 2011). *Campylobacter* phages have been isolated from several different sources such as sewage, pig and poultry manure, abattoir effluents, broiler chickens and retail poultry (Carrillo *et al*., 2005). A study has reported that under experimental conditions, the use of a phage cocktail was able to reduce the level of colonization of *Campylobacter* in infected poultry by approximately 2 $\log_{10}$cfu/g (Carvalho *et al*., 2010). The application of phage therapy as part of poultry farming practices and the costs associated with this process are not known (Kaakoush *et al*, 2015). Bacteriocins are antimicrobial peptides that are lethal to bacteria other than the producing strain, and are biosynthesized by a wide variety of Gram-positive and Gram-negative bacteria (Gillor *et al*., 2008). A study revealed that administration of three bacteriocins (OR-7 from *Lactobacillus salivarius* and E-760 and E50-52 from *Enterococcus faecium*) to broiler chickens resulted in reductions of 5 to 8 $\log_{10}$ CFU in *C. jejuni* intestinal colonization. Thus, bacteriocins may be promising agents for the control of *C. jejuni* in commercial broiler chickens (Kaakoush *et al*, 2015).

The cross-contamination of broiler carcasses by spilled gut contents and the cross-contamination from *Campylobacter*-positive to *Campylobacter*-negative revealed a potential hygiene problem in slaughterhouse, showing that standard biosecurity measures on the processing plants are ineffective.

At the processing level, measures to decontaminate broiler chicken carcasses include physical and chemical methods, but their effectiveness is typically limited to a reduction of 1–2 log units (Wagenaar *et al*., 2013). Treatment with chemical agents, such as chlorine dioxide (CD), acidified sodium chlorite (ASC), trisodium phosphate (TSP) and peroxyacetic acid (PAA) in washing steps, cetylpiridinium chloride and propylene glycol for poultry carcass dipping, and peroxyacetic acid solutions, acetic acid, or lactic acid to decontaminated carcasses, would help to reduce partly the number of *Campylobacter* (Klein, 2016). In European Union only use of drinking water was permitted to disinfect meat carcasses; very recently the EU legislation allowed lactic acid to decontaminate beef carcasses. Other treatments, including PAA or chlorine for poultry have not been approved in the EU (BEUC, 2015). In the United States, organic acids, quaternary ammonium compounds, acidified sodium chlorite, and trisodium phosphate are being applied as decontaminating agents in practices (Wagenaar *et al*., 2013).

Irradiation is the most effective physical decontamination method used to remove *Campylobacter* from broiler chicken meats (Toldrá, 2009). The DNA of the cell is the most critical target of ionizing radiation, and the inactivation of microorganisms is primarily due to damage to the DNA. The safety of irradiated foods for human consumption has been questioned because ionizing radiation can lead to chemical changes. The Food and Drug Administration (FDA) and United States Department Agriculture (USDA) have approved irradiation of broiler chickens at a maximum dose of 3 kGy to control foodborne pathogens, such as *Campylobacter* (Keener *et al*., 2004). EFSA's esperts revealed that the substances formed in food by irradiation are also formed during other types of food processing, with levels comparable to heat treatment of foods. However, consumers do not like and do not accept irradiated products (EFSA, 2011a).

1.4.5 *Campylobacter* survival in broiler chicken products

Poultry is a highly perishable food. After slaugher, raw broiler chicken products tends to deteriorate in 4-10 days even when stored under chilled conditions (Meredith *et al*., 2014). *Campylobacter* spp are very sensitive to freezing, heat, desiccation, low water activity, UV light and salt. *Campylobacter* is unable to grow and multiply at the temperatures normally used to store food. Survival at room temperature is poor; in particular, heat treatments and the freezing-thawing inactivated and reduced, respectively, the population of *Campylobacter* spp. *Campylobacter* is heat sensitive and do not survive also at moderate cooking (Silva *et al*., 2011). Refrigeration and freezing are interventions used in the control of bacterial growth in foods (Bhaduri and Cottrell, 2004) but the freezing does not eliminate the pathogens from contaminated food (Silva *et al*., 2011). Counts of *C. jejuni* on poultry carcasses decline during refrigerated or freezing storage (Bhaduri and Cottrell, 2004; Alter *et al*., 2005; Eideh and Al-Qadiri, 2011), though a portion of a *C. jejuni* population can survive on raw or cooked poultry samples during refrigeration or freezing (Bhaduri and Cottrell, 2004; Eideh and Al-Qadiri, 2011; Maziero and De Oliveira, 2010). Many factors can cause injury or death of *Campylobacter* cells such as ice nucleation, dehydration or oxidative stress. After chilled or frozen storage is needed control and detect cell's viability (Maziero and De Oliveira, 2010).

Modified atmosphere packaging (MAP) has received increasing attention in the food industry as a method for food preservation, to extend the shelf-life of fresh and processed chilled broiler chicken meats by inhibition fast-growing aerobes and by control spoilage microorganism such as

Pseudomonas (Meredith *et al*., 2014). In particular, MAP has been defined by Parry (1993) as the "enclosure of food products in high gas barrier materials, in which the gaseous environment has been changed or modified to slow respiration rates, reduce microbiological growth and retard enzymatic spoilage, with the intent of extending shelf life". MAP is a non-thermal method of food preservation that uses three gases, nitrogen (N_2), oxygen (O_2) and carbon dioxide (CO_2), in various ratios. These gases can be applied individually or in combination to have different purposes in food preservation (Rao and Sachindra, 2002). O_2 is important in the storage of fresh broiler chicken meats as it maintains the meat pigment myoglobin in its oxygenated form, oxymyoglobin, which keeps the bright red colour of fresh meat, which consumers prefer (Šuput *et al*., 2013). O_2 promotes the growth of aerobic bacteria and inhibits the growth of facultative anaerobic or anaerobic bacteria (Meredith *et al*., 2014). If exposed to O_2, *C.jejuni* cells became slightly elongated, less coiled and lose their motility. The loss of spiral morphology has been reported to be one of the stages before coccoid formation, which, among others, has been associated with oxidative stress and limited nutrients (Boysen *et al*., 2007). N_2 is an inert gas, with no antimicrobial activity, used to displace the O_2 in the packs and storage vessels to delay oxidative rancidity and inhibit the growth of aerobic microorganisms. Its primary function is as a filler and to prevent the collapse of the pack containing high concentration of CO_2 (Sanjeev and Ramesh, 2006). Carbon dioxide (CO_2) is an active gas, highly soluble in water and fat tissues with bacteriostatic properties, inhibiting the increase of most aerobic bacteria; thus it is the most important gas in the packaging of food under modified atmospheres (Meredith *et al*., 2014). Although the specific way in which CO_2 exerts its bacteriostatic effect is unknown, the overall effect on microorganisms is an extension of the lag phase of growth and a decrease in the growth rate during the logarithmic growth phase.

Carbon dioxide acts on bacterial cell decreasing intracellular pH and reducing enzyme activity, inhibiting decarboxylating and nondecarboxylating enzymes and altering membrane properties with consequent inhibition of membrane functions (Meredith *et al*., 2014).

There are study which showed that CO_2 can have a protective effect on *Campylobacter* strains, promoting it survival and protecting it from the negative effect of oxygen (Boysen *et al*.,2007; Byrd *et al*., 2011; Meredith *et al*., 2014).

1.5 Aims and objectives of this thesis

Broiler chickens represent an important sector in animal production in Italy. Broilers are considered the major reservoir for *Campylobacter* spp and are responsible of most cases of human campylobacteriosis.

The aim of this thesis was to evaluate *Campylobacter* contamination along broiler chicken chain from farm to slaughterhouse and evaluate the effect of packaging and refrigeration on survival of *Campylobacter* on broiler chicken products at end shelf-life.

More specifically the main aims were to:

- investigate the presence of *Campylobacter* spp in cloacal swabs in broiler chicken farm and in neck skins of carcasses at slaughterhouse to evaluate a possible correlation between these two environments;
- investigate the presence and the concentration of *Campylobacter* spp in broiler chicken products, with skin and skinless, collected within 1 h after slaughter;
- identify at level of species using a multiplex PCR related strains of the genus *Campylobacter* previously isolated from broiler farms and from broiler carcasses;
- investigate the genetic diversity and antimicrobial resistance of *C. jejuni* and *C.coli* isolates obtained from broiler farms and from broiler carcasses;
- estimate the effect of refrigeration storage during the time in two different packaging types on the survival of *C. jejuni* and *C. coli* in the broiler chicken products;

- investigate the presence of virulence genes (*Cdt*), including *cdt*A, *cdt*B, and *cdt*C, only in *C. jejuni* strains isolated from broiler chicken meats at end of shelf-life period.

Also the analysis of antibiotic resistance and molecular by PFGE were carried out in collaboration with the Department of Veterinary Medicine of University of Perugia and with Istituto Zooprofilattico of Abruzzo and Molise (IZS Teramo), national reference center for *Campylobacter*. All *Campylobacter* isolated in this thesis were recorded to help to a better understanding of the epidemiology of *Campylobacter* along the chain production of broiler chickens in Italy.

CHAPTER 2. Presence, genetic diversity and antimicrobial resistance of *Campylobacter* spp strains isolated from broiler chickens farms and at slaughterhouse in central Italy

2.1 Introduction

Broiler chickens are frequently colonized in the intestinal tract with high numbers of *Campylobacter*, primarily *C. jejuni* and *C. coli*. Colonized broilers entering the slaughter line can cause cross-contamination within the slaughterhouse environment and lead to *Campylobacter* free carcasses contamination (Vidal *et al*., 2016). Particularly, scalding, defeathering, evisceration and giblet operations are major points of transfer of pathogens in poultry processing (Chantarapanont *et al*, 2003). The high genetic diversity observed among *Campylobacter* strains isolated from commercial broiler chickens in farms has been demonstrated in several different studies, using different genotyping methods (Rivoal *et al*., 2005; Wittwer *et al*., 2005; Johnsen *et al*., 2007; Alter *et al*., 2011; Giacomelli *et al*., 2012; Marotta *et al*., 2015; Vidal *et al*., 2016).

Macrolide and quinolone resistance in *Campylobacter* strains is actually recognized as an emerging public health problem. Moreover, it was shown that patients infected with macrolide or quinolone resistant *Campylobacter* isolates could be associated with increased risk of adverse events or development of the invasive form of the disease compared with patients infected with macrolide or quinolone susceptible isolates (Lehtopolku *et al*., 2010).

The aim of this study was to investigate the presence of *Campylobacter* spp along broiler chickens chain, particularly in live broiler chickens belonging from twenty-two farm, and on chilled carcasses, after slaughter. All broiler chickens were processed in the same slaughterhouse. Furthermore, in the present study diversity genetic and antibiotic susceptibility of *Campylobacter* isolated from cloacal swabs (n=116) on eight farms analyzed and from neck skins of chilled carcasses (n=24) at slaughterhouse, coming from the same farms, were investigated.

2.2 Material and Methods

2.2.1 Sampling

The investigation was conducted in years 2014 and 2015 in twenty-two broiler chicken farms for poultry production randomly selected in the center of Italy. The farms tested had a conventional indoor broiler production system and the size of the flocks tested ranged from 10.000 to 25.000 broiler chickens (Ross 708). All birds were vaccinated following the traditional plain vaccination (Marek's disease, Newcastele disease and coccidiosis) and had not been submitted to any antimicrobial treatment during the production cycle. All farms were visited once and sampling consisted in the collection of fresh feces from the cloaca of 28 random broiler chickens using a sterile cotton swab. A total of 616 cloaca swabs was taken. After collection, each swab was immediately placed in sterile Amies transport medium with charcoal (Oxoid), transported to the laboratory within 20 min, and processed immediately. Slaughter age of studied broiler flocks ranged from 38 to 42 days. A second step of *Campylobacter* strains isolation was made in the slaughterhouse. In this case, 15 broiler chicken carcasses, coming from the same farms previously

analyzed, were used to collect a portion of neck skins at the end of the processing line after chilling. A total of 330 skin samples were taken and transferred directly in sterile stomacher bags, transported to the laboratory and subjected to analysis within four hours. The weight of skin samples ranged from 20.0 to 25.0g.

2.2.2 Isolation and identification of *Campylobacter* spp

The samples were processed according to the method described in ISO 10272-1:2006.

Cloacal swabs were placed in plastic tubes with 5 ml of selective enrichment broth, Bolton Broth (Oxoid), containing 5% lysed horse blood, vortexed for 30s and incubated at 41.5 ± 0.5°C for 48 hours in microaerophilic conditions (CampyGen, Oxoid) provided by commercial gas packs.

In parallel, each neck skins obtained from the slaughterhouse was added to 200-225 mL of Bolton Broth (Oxoid), with 5% lysed horse blood, homogenized on the stomacher and incubated in microaerophilic conditions (CampyGen, Oxoid) at 41.5 ± 0.5°C for 48 hours.

After incubation, 10-µl loop-full of broth was then streaked onto modified charcoal cefoperazone deoxycholate agar (mCCD agar, Oxoid) and incubated under microaerophilic conditions (CampyGen, Oxoid) at 41.5 ± 0.5°C for 48 hours. For each positive plate, two or three presumptive *Campylobacter* colonies (greyish, metallic sheen, flat, moist with tendency to spread) (Fig. 6) were subculturated on nonselective Columbia blood agar (Oxoid) (Fig. 7) and incubated under microaerophilic conditions (CampyGen, Oxoid) at 41.5 ± 0.5°C for 48 hours. Isolates were confirmed by biochemical tests (oxidase test, catalase test, hippurate hydrolysis test), microscopic examination and gram stained (Tab. 4). Colonies from Columbia blood agar were stored in Brucella broth (Biolife) supplemented with 30% glycerol at -80°C to await further analysis.

Morphology	Small curved bacilli
Motility	Characteristic (mobile with rapid movements or corkscrew)
Microaerophilic growth at 25°C	-
Aerobic growth at 42°C	-
Oxidase	+
Catalase	+

Table 4. Microbiological testing summary

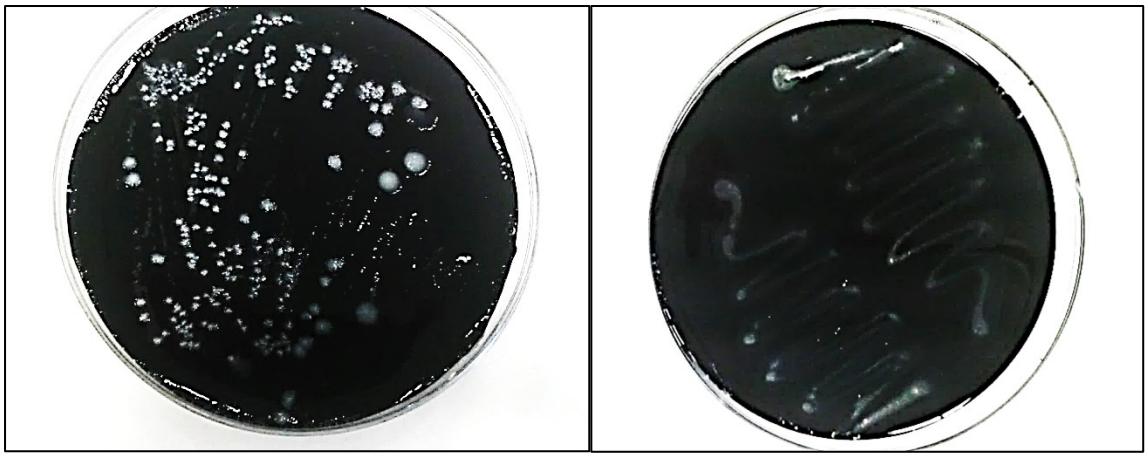


Figure 6. Growth of *Campylobacter* colonies on mCCD agar.

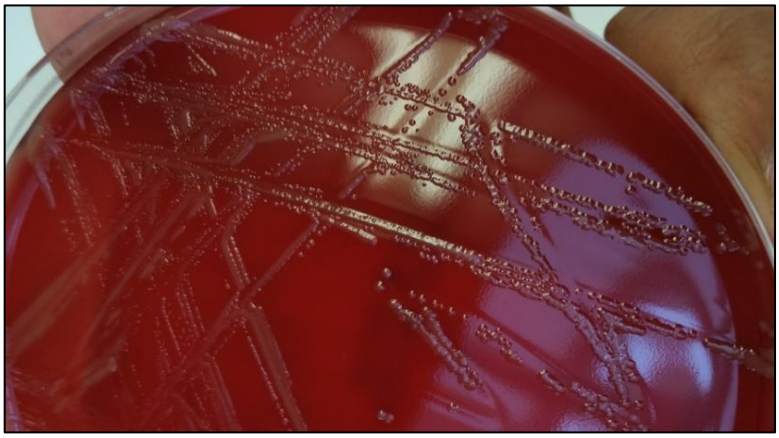


Figure 7. Growth of *Campylobacter* colonies on Columbia Blood Agar

2.2.3 DNA extraction and molecular identification of *Campylobacter* genus and species

Strains phenotypically classified as *Campylobacter* were identified by conventional multiplex PCR assay, as previously described by Wang *et al* (2002). The strains used as positive controls were *Campylobacter coli* NCTC 11353, *Campylobacter fetus subsp.fetus* ATCC 27374, *Campylobacter jejuni* ATCC 33291, *Campylobacter upsaliensis* NCTC 11541 and *Campylobacter lari* NCTC 11552. Chromosomal DNA was extracted using an Ultraclean Microbial DNA Isolation kit (MO BIO Laboratories), quantified using a Nanodrop Spectrophotometer and DNA concentration was adjusted to approximately 50 ng/µl for each sample. Six pairs of primers were used to identify the genes *hipO* from *C. jejuni*; *glyA* from *C. coli*, *C. lari*, and *C. upsaliensis*; *sapB2* from *C. fetus* subsp. *fetus*; and the internal control 16S rRNA. The multiplex PCR amplification was performed in a final volume of 50µl containing 5µL of 10X buffer, 200µM of each deoxynucleoside triphosphate, 1 U of *Taq* DNA polymerase, 0.5 µM *C. jejuni* and *C. lari* primers; 1 µM *C. coli* and *C. fetus* primers, 2 µM *C. upsaliensis* primers and 5µL of DNA. The DNA extracted from the samples under analysis was used as template for amplification which provided an initial denaturation step at 95°C for 5 min followed by 30 cycles of amplification (denaturation at 95°C for 30 sec, annealing at 59°C for 30 sec, and extension at 72°C for 30 sec), with a final extension at 72°C for 7 min. The PCR products were detected on a 1.5% agarose gel, stained with 4 µL of ethidium bromide, run at 78 V for 40 min, visualized under UV light and photographed at the transilluminator.

The 16S PCR amplification was performed in a final volume of 50µl containing 5µL of 10X buffer, 200µM of each deoxynucleoside triphosphate, 1 U of *Taq* DNA polymerase, 1 µM of primers *Campylobacter* and 5µL of DNA. The amplification provided an initial denaturation step at 95°C for 5 min followed by 30 cycles of amplification (denaturation at 95°C for 30 sec, annealing at

52°C for 30 sec, and extension at 72°C for 30 sec), with a final extension at 72°C for 7 min. The PCR products were detected on a 1.5% agarose gel, stained with 4 μL of ethidium bromide, run at 78 V for 40 min, visualized under UV light and photographed at the transilluminator.

Primer	Sequence (5'-3')	Target gene	Microorganism
CJF (25pm) CJR (25pm)	ACTTCTTTATTGCTTGCTGC GCCACAACAAGTAAAGAAGC	*hipO*	*C. jejuni*
CCF (50pm) CCR (50pm)	GTAAAACCAAAGCTTATCGTG TCCAGCAATGTGTGCAATG	*glyA*	*C. coli*
CLF (25pm) CLR (25pm)	TAGAGAGATAGCAAAAGAGA TACACATAATAATCCCACCC	*glyA*	*C. lari*
CUF (100pm) CUR (100pm)	AATTGAAACTCTTGCTATCC TCATACATTTTACCCGAGCT	*glyA*	*C. upsaliensis*
CFF (50pm) CFR (50pm)	GCAAATATAAATGTAAGCGGAGAG TGCAGCGGCCCCACCTAT	*sapB2*	*C. fetus*
16S 1a (50pm) 16S 1b (50pm)	AATACATGCAAGTCGAACGA TTAACCCAACATCTCACGAC	16S	*Campylobacter* *Helicobacter* *Arcobacter*

Table 5. Primer sequences used in the multiplex and 16S PCR assay

Initial denaturation	Amplification cycles = 30			Extension cycle	Final cycle
	denaturation	**annealing**	**extension**		Tempo ∞
95°C x 5'	95°C x 30''	59°C x 30"	72°C x 30''	72°C x 7'	4°C

Table 6. Amplification program of the multiplex PCR

Initial denaturation	Amplification cycles = 30			Extension cycle	Final cycle
	denaturation	annealing	extension		Tempo ∞
95°C x 5’	95°C x 30’’	52°C x 30”	72°C x 30’’	72°C x 7’	4°C

Table 7. Amplification program of the 16S PCR

	Microorganism	Target gene	Size amplified (bp)
Multiplex PCR	*C. coli*	*glyA*	126
	C. upsaliensis	*glyA*	204
	C. lari	*glyA*	251
	C. jejuni	*hipO*	323
	C. fetus sub. fetus	*sapB2*	435
16S PCR	*Campylobacter spp* *Helicobacter spp* *Arcobacter spp*	16S	1125

Table 8. Predicted PCR amplicon size (in bp)

2.2.4. s-AFLP typing

Representative strains of *Campylobacter jejuni* and *Campylobacter coli* isolated from eight farms were typed using the s-AFLP (single-enzyme amplified fragment length polymorphism) technique as described by Champion *et al*. 2002. s-AFLP is a technique capable of genotyping genomic DNA from any origin. The method of genotyping bacteria by s-AFLP was developed by Vos *et al*. (1995). This technique involves the digestion with a single enzyme, resulting in binding of the fragments to specific oligonucleotides that amplified by PCR generate to typical profiles for each strain. The extracted DNA from each strain was digested with the restriction endonuclease *Hind*III in a total volume of 20µl containing 1µl of spermidine, 2µl of 10X buffer, and 2µl of *Hind*III at 37°C overnight. The digested DNA was subjected to ligation for 3 h at room temperature in a total volume of 20µl containing 5µl of digested DNA, nuclease-free water, 4µl of 5X ligase buffer, 50µM of Adapter H1 (5' ACG GTA TGC CAC AG 3'), 50µM of Adapter H2 (5' AGC TCT GTC GCA TAC CGT GAG 3') and 1U of T4 DNA ligase. Digested and ligated DNA was incubated at 80°C for 10 minutes to inactivate the ligase and further amplified with an amplification mixture containing 50µl of 5µL of 10X buffer, 200µM of each deoxynucleotide triphosphate (dNTP), 2.5 mM MgCl2 , 1.5µM primer HI-C (5' GGT ATG CGA CAG AGC TTC 3'), 1 U of *Taq* DNA polymerase and 5µL of digested and ligated DNA. The amplification cycle includes an initial denaturation at 94 °C for 5 min, followed by 33 cycles of amplification (94°C for 1 min, 60°C for 1 min and 72°C for 2.5 min) ending with a cycle of extension at 72° C for 7 min. The PCR products were detected in a 2.5% agarose gel, stained with 4 µL of ethidium bromide, run at 68V for 3 h, visualized under UV light, photographed and analyzed.

2.2.5 Antimicrobial susceptibility testing

Susceptibilities to antibiotics of representative strains of *Campylobacter jejuni* and *Campylobacter coli*, isolated from eight farms, were evaluated by MIC. Colonies were picked and grown on Columbia agar (Oxoid) at 42°C in a microaerophilic atmosphere for 24 h. Next, the colonies were seeded in Mueller Hinton Broth supplemented with blood and dispensed into Eucamp microtitre plates containing known scalar concentrations of the following antibiotics: gentamicin (0.12–16 μg/ml), streptomycin (1–16 μg ml^{-1}), ciprofloxacin (0.06–4 μg ml^{-1}), tetracycline (0.25–16 μg $ml^{-1)}$, erythromycin (0.5–32 μg ml^{-1}), nalidixic acid (2–64 μg ml^{-1}) and chloramphenicol (2–32 μg ml^{-1}). After inoculation, the plates were incubated at 42°C in a microaerophilic atmosphere for 24 h and then screened. *Campylobacter jejuni* strain NCTC 11351 was used as the control. The results for chloramphenicol were evaluated according to the interpretation criteria of the French Society of Microbiology, while breakpoints established from EUCAST for *Campylobacter* spp. were used for all other antibiotics.

2.2.6 Statistical Analysis

Statistical analysis software JMP SAS was used to obtain an estimation of *Campylobacter jejuni* and *Campylobacter coli* populations in broiler chickens and to represent graphically the distance among the resulting genotypes. Initially, AFLP fragments were scored manually for the presence (1) or absence (0) of bands to create a binary matrix. The binary data matrix was analysed with the JMP SAS program to calculate the Euclidean distance and get the distance matrix. Particularly, measurements of genetic distances that reflect the genetic similarity between *C. jejuni* and *C. coli* strains were obtained with cluster analysis. Ward's method was used as the method of cluster formation to obtain a final output dendrogram of connection. Descriptive statistics were used to summarize antibiotic susceptibility of the *C. jejuni* and *C. coli* isolates according to their source (cloacal and carcass samples). The relationship between antibiotic susceptibility and bacterial species was evaluated by the chi-square Fisher's exact tests. Association among farm, antibiotic resistance and AFLP pattern was evaluated by binary logistic regression. The chi-square of the overall model and the associated P-values were reported. Data were analyzed using SPSS Statistics, version 20 (IBM, SPSS Inc., Chicago, IL) and $P < 0.05$ was the accepted level of statistical significance.

2.3 Results

From April 2014 untill July 2015, a total of 616 cloacal swabs and 330 neck skins, belonging from twenty-two different farms, were sampled in farm and in slaughterhouse, respectively, to determine *Campylobacter* colonization on farms and the cross-contamination in slaughterhouse.

Genetic diversity and antimicrobial resistance of *C. jejuni* and *C. coli* strains, isolated from eight farms, was investigated.

2.3.1 Presence of *Campylobacter* spp colonization in broiler chickens

2.3.1.1 Isolation and identification

In this study, out of twenty-two broiler chicken farms monitored, eleven (50%) farms were colonized with *Campylobacter* spp, based on cloacal swabs samples collected on-farm (Fig. 8; Table 9).

The presence of *Campylobacters* spp in processed chilled broiler chickens, tested by neck skin samples, was 36%, with *Campylobacter* present on the carcasses of five broiler chicken farms, previously identified as *Campylobacter*-positive from on-farm fecal samples. Also, *Campylobacters* were found on the carcasses of three broiler chicken farms, identified as *Campylobacter*-negative, before slaugher, from on-farm fecal samples (Fig. 8; Tab. 9).

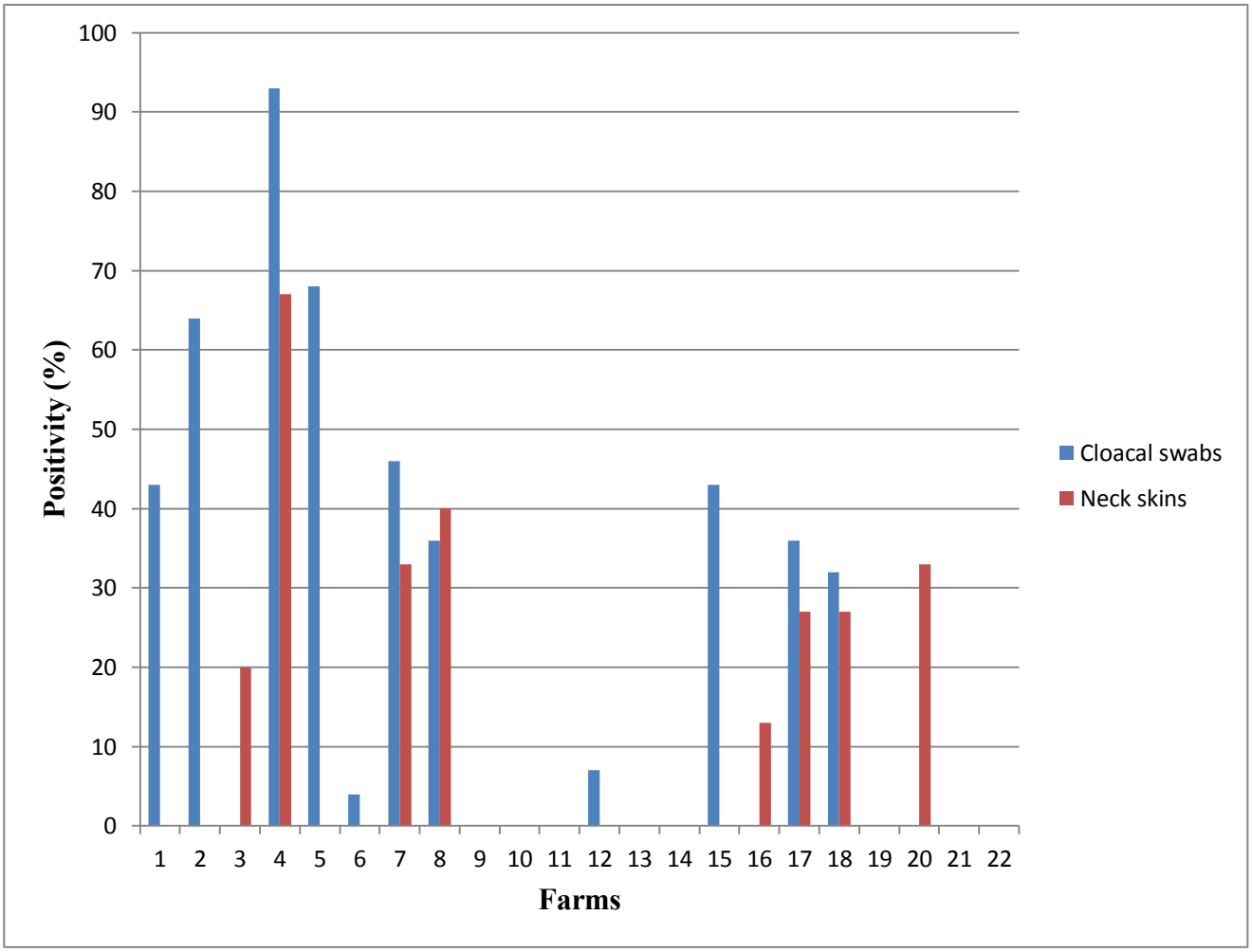


Figure 8. Percentage of positive cloacal swabs samples and neck skin samples

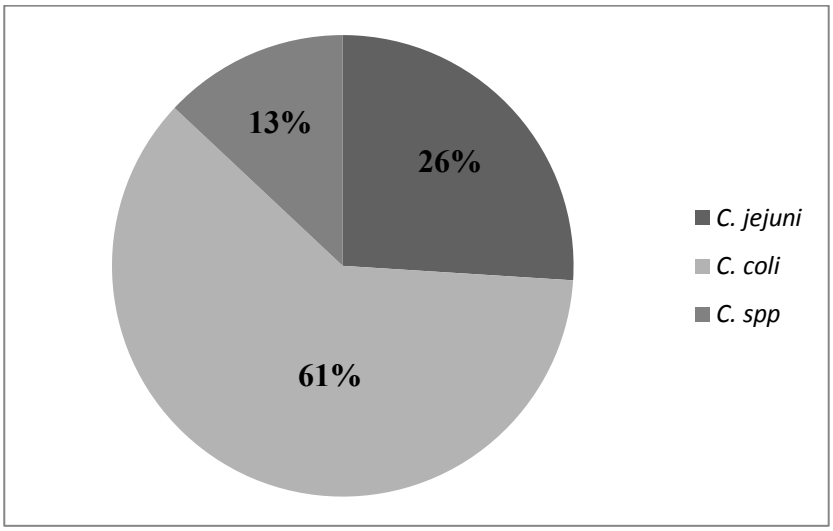
Of the twenty-two broiler chicken farms analyzed in this study, testing cloacal swab samples in live birds, 1 had only the *C. jejuni* species, 4 had only *C. coli*, and 4 were infected by both species; of the broiler chicken carcasses, coming from the same twenty-two farms previously analyzed, instead, 1 had only the *C. jejuni* species, 6 had only *C. coli*, and 1 were infected by both species (Tab. 9). Most of the carcasses were contaminated with the same *Campylobacter* species as identified in the corresponding farm before slaughter (Tab. 9).

At a farm level, the species distribution of all broiler chickens *Campylobacter* isolates was 26% *C. jejuni*, 61% *C. coli* and 13% *C. spp* (Tab. 9; Graph. 1); this distribution was similar between strains isolated from slaughterhouse (17% *C. jejuni*; 83% *C. coli*) (Tab. 9; Graph. 2).

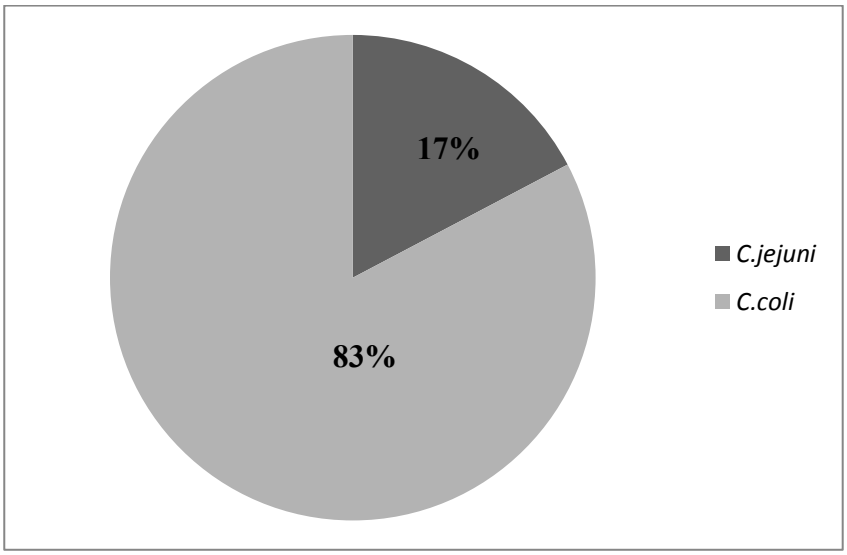
Farm	Cloacal swab samples				Neck skin samples			
	No of positive sample/Total (%)	Species Identification (%)			No of positive sample/Total (%)	Species Identification (%)		
		C.jejuni	*C.coli*	*C.spp*		*C.jejuni*	*C.coli*	*C.spp*
1	12/28 (43%)	24	0	0	0/15 (0%)	0	0	0
2	18/28 (64%)	0	36	0	0/15 (0%)	0	0	0
3	0/28 (0%)	0	0	0	3/15 (20%)	0	11	0
4	26/28 (93%)	0	52	0	10/15 (67%)	0	20	0
5	19/28 (68%)	32	6	0	0/15 (0%)	0	0	0
6	1/28 (4%)	1	1	0	0/15 (0%)	0	0	0
7	13/28 (46%)	0	0	26	5/15 (33%)	0	10	0
8	10/28 (36%)	0	20	0	6/15 (40%)	0	12	0
9	0/28 (0%)	0	0	0	0/15 (0%)	0	0	0
10	0/28 (0%)	0	0	0	0/15 (0%)	0	0	0
11	0/28 (0%)	0	0	0	0/15 (0%)	0	0	0
12	2/28 (7%)	1	1	2	0/15 (0%)	0	0	0
13	0/28 (0%)	0	0	0	0/15 (0%)	0	0	0
14	0/28 (0%)	0	0	0	0/15 (0%)	0	0	0
15	12/28 (43%)	0	18	6	0/15 (0%)	0	0	0
16	0/28 (0%)	0	0	0	2/15 (13%)	0	2	0
17	10/28 (36%)	0	20	0	4/15 (27%)	8	0	0
18	9/28 (32%)	12	6	0	4/15 (27%)	6	2	0
19	0/28 (0%)	0	0	0	0/15 (0%)	0	0	0
20	0/28 (0%)	0	0	0	5/15 (33%)	0	10	0

21	0/28 (0%)	0	0	0	0/15 (0%)	0	0	0
22	0/28 (0%)	0	0	0	0/15 (0%)	0	0	0
Total	**132/616 (21%)**	**70 (26%)**	**160 (61%)**	**34 (13%)**	**39/330 (12%)**	**14 (17%)**	**67 (83%)**	**0 (0%)**

Table 9. Presence *Campylobacter* in cloacal swabs and in neck skins



Graphic 1. Proportions of *C. jejuni* and *C. coli* identified among *Campylobacter* spp strains (n=264), isolated from on farm broiler chickens



Graphic 2. Proportions of *C. jejuni* and *C. coli* identified among *Campylobacter* spp strains (n=81), isolated from chilled broiler chicken carcasses

2.3.2 s-AFLP typing

Once a sample proved positive, one single colony of *Campylobacter* spp. was taken for further characterization.

Particularly, a total of 140 *Campylobacter* strains isolated from eight broiler chickens farms analyzed (1, 2, 4, 5, 8, 15, 17, 18), obtained from cloacal swabs (n = 116) and from neck skins of processed broiler chicken carcasses (n = 24) (Tab. 10), were characterized by single enzyme amplified fragment length polymorphism (s-AFLP) and by antimicrobial susceptibility testing.

Farm	**Sources**	***C. jejuni* (n=41)**	***C. coli* (n=99)**	**Total (n=140)**
	CS	12	0	
1	CA	0	0	12
	CS	0	18	
2	CA	0	0	18
	CS	0	26	
4	CA	0	10	36
	CS	16	3	
5	CA	0	0	19
	CS	0	10	
8	CA	0	6	16
	CS	0	12	
15	CA	0	0	12
	CS	0	10	
17	CA	4	0	14
	CS	6	3	
18	CA	3	1	13

Table 10. *C. jejuni* and *C. coli* isolates recovered from cloacal swabs (CS) and chilled carcass samples (CA)

The s-AFLP *Campylobacter jejuni* isolates were grouped into four different genotype profiles (banding patterns a-d), while *Campylobacter coli* isolates were grouped into seven different genotype profiles (banding pattern e-m) (Tab. 11).

Source	*Campylobacter* species	N. Banding patterns (CS)*	N. Banding patterns (CA)*
Farm 1	*C. jejuni*	12 a	
Farm 2	*C. coli*	18 e	
Farm 4	*C. coli*	26 f	10 f
Farm 5	*C. coli*	3 g	
Farm 5	*C. jejuni*	9 b, 7 c	
Farm 8	*C. coli*	10 h	6 h
Farm 15	*C. coli*	12 i	
Farm 17	*C. coli*	10 l	
Farm 17	*C. jejuni*		4 d
Farm 18	*C. coli*	3 m	1 m
Farm 18	*C. jejuni*	6 d	3 d

* CS cloacal swabs; CA carcasses samples

Table 11. s-AFLP types of 41 *C. jejuni* and 99 *C. coli* isolates.

The cluster analysis based on the dendrogram generated from s-AFLP, with a 70% similarity level, demonstrated that *C. jejuni* isolates were grouped into four clusters (Fig. 9) while the *C. coli* isolates were grouped into six cluster (Fig. 10).

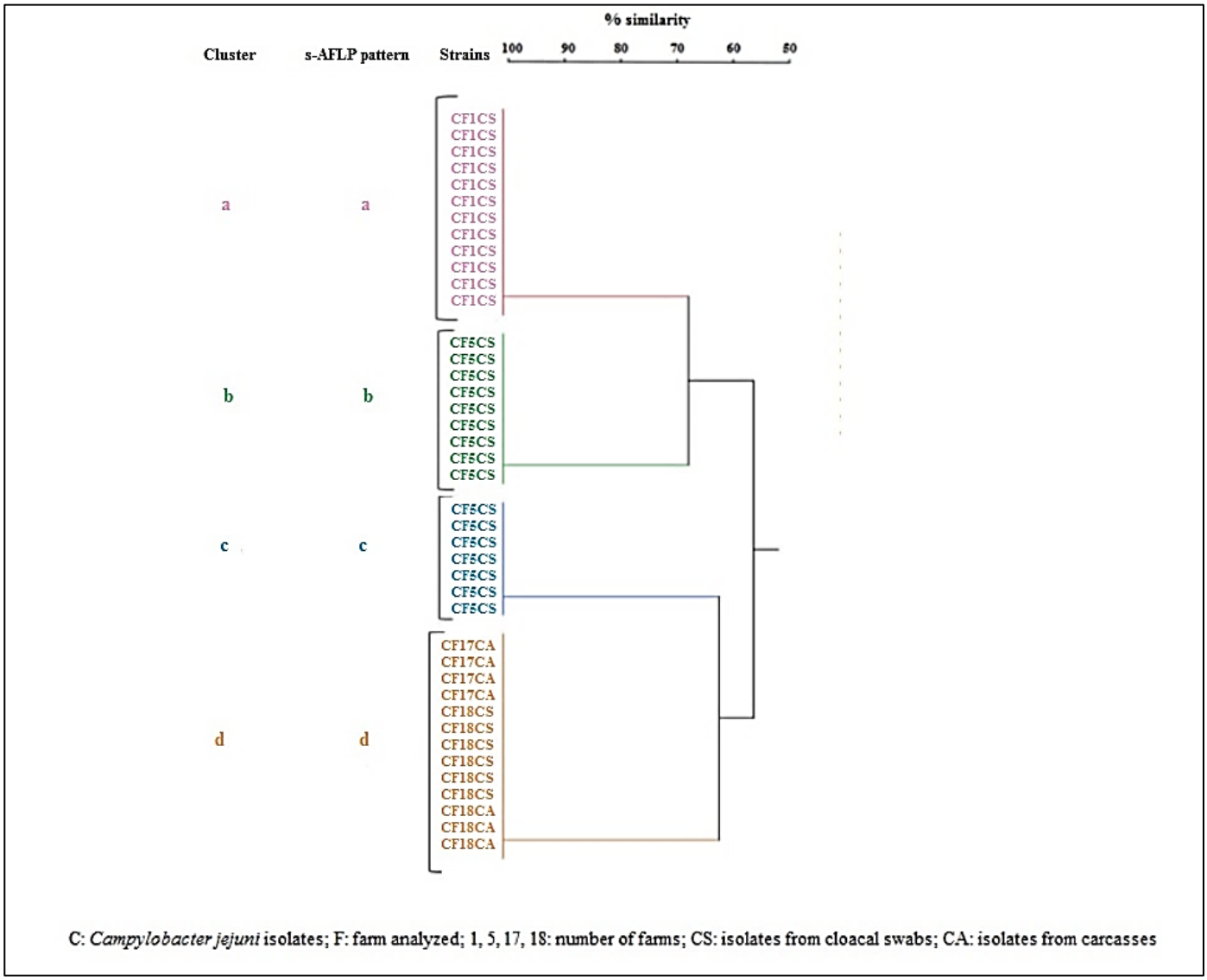
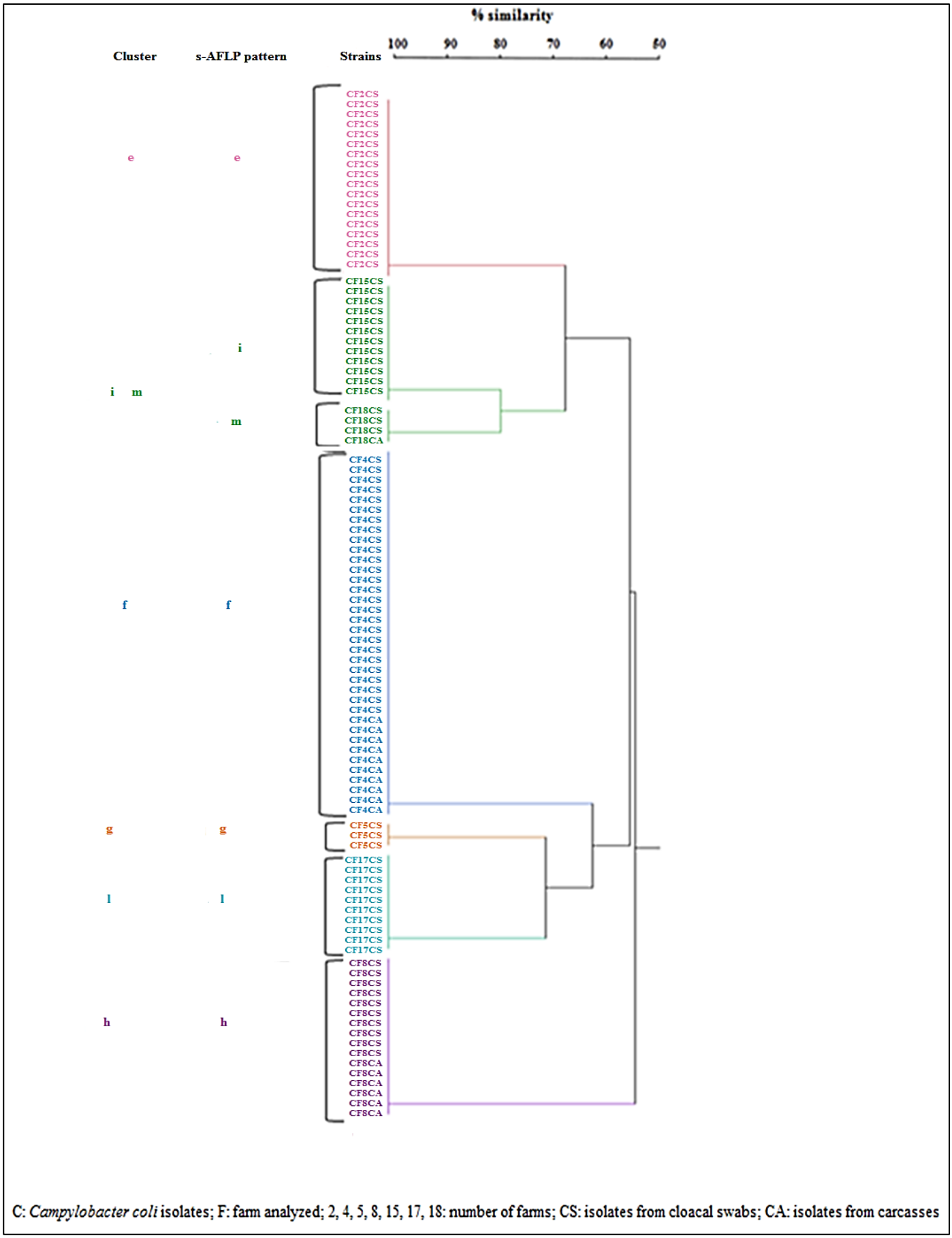


Figure 9. Dendrogram of s-AFLP profiles of 41 *C. jejuni* strains



C: *Campylobacter coli* isolates; F: farm analyzed; 2, 4, 5, 8, 15, 17, 18: number of farms; CS: isolates from cloacal swabs; CA: isolates from carcasses

Figure 10. Dendrogram of s-AFLP profiles of 99 *C. coli* isolates

The s-AFLP types of both *C. jejuni* and *C. coli* were strongly associated with the farm ($P < 0.0001$). Indeed, the s-AFLP patterns of *C. coli* isolates within the same farm emphasized the presence of characteristic s-AFLP genotypes (Fig. 10). In the case of *C. jejuni*, the isolates from farms 17 and 18 showed the same genotype profile even though they were derived from two different farms. The s-AFLP typing of *C. jejuni* also revealed the presence of two different patterns and clusters within farm 5, indicating genetic diversity among *C. jejuni* isolates (Fig. 9). Moreover the s-AFLP genotypes of *C. jejuni* and *C. coli* found in the cloacal swabs were the same detected from the cutaneous samples collected from the subjects coming from the same farms analyzed.

2.3.3 Antimicrobial Susceptibility testing

Higher prevalence of resistance were observed in *C. coli* compared to *C. jejuni* for ciprofloxacin ($P < 0.01$), nalidixic acid ($P < 0.001$), tetracycline ($P < 0.001$) and erythromycin ($P < 0.001$) (Tab. 12).

		N° isolates (%)						
		GM	ST	CIP	TE	E	NA	C
C. coli	R	0(0)	*0(0)	*69(70)	*69(70)	*30(30)	*69(70)	0(0)
C. coli	S	99(100)	99(0)	30(30)	30(30)	69(70)	30(30)	99(100)
C. jejuni	R	0(0)	*12(29)	*16(39)	*4(10)	*0 (0)	*16(39)	0(0)
C. jejuni	S	41(100)	29(71)	25(61)	37(90)	41(100)	25(61)	41(0)

GM, gentamicin; ST, streptomycin; CIP, ciprofloxacin; TE, tetracycline; E, erythromycin; NA, nalidixic acid; C, chloramphenicol

*Statistically significant difference between *C. coli* and *C. jejuni* isolates ($P < 0.01$, $P < 0.001$)

Table 12. Antibiotic resistance profiles of *C. coli* and *C. jejuni* isolates.

Conversely, the rate of streptomycin resistance was higher in *C. jejuni* than *C. coli* ($P < 0.001$). Combined resistances for ciprofloxacin, nalidixic acid and tetracycline were observed in *C. jejuni*, as well as for ciprofloxacin and nalidixic acid. Moreover 13 out of 41 isolates (32%) were completely susceptible and 12 out of 41 isolates (29%) were resistant to streptomycin. Furthermore these phenotypes were strictly correlated to s-AFLP genotypes ($P < 0.001$) as shown in Figure 11.

Multiple resistances were common in *C. coli*, with two patterns being recognized: either strains were resistant for ciprofloxacin, nalidixic acid and tetracycline, or for ciprofloxacin, nalidixic acid, tetracycline and erythromycin. Single resistance was not observed and 30% of *C. coli* isolates were completely susceptible. These three phenotypes were also strictly correlated to s-AFLP patterns ($P < 0.001$), as shown in Figure 12. In addition, the *C. jejuni* and *C. coli* antibiotic phenotypes were also correlated to farms ($P < 0.001$) (Figures 11, 12).

Moreover there were no differences in the pattern of resistance of *C. jejuni* and *C. coli* strains isolated from fecal and neck skin samples of the same farm (Fig. 11, 12).

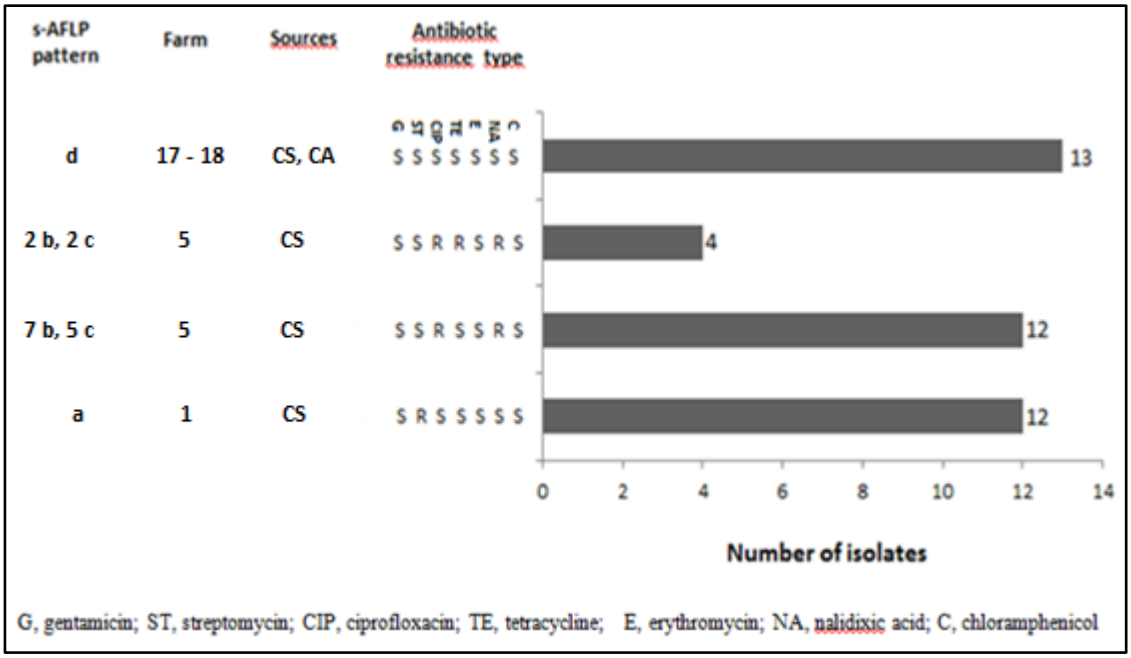


Figure 11. Antimicrobial resistance profiles and s-AFLP patterns of *C. jejuni* isolated from cloacal swabs (CS) on farm and carcass samples (CA) at the end of the processing line, after chilling

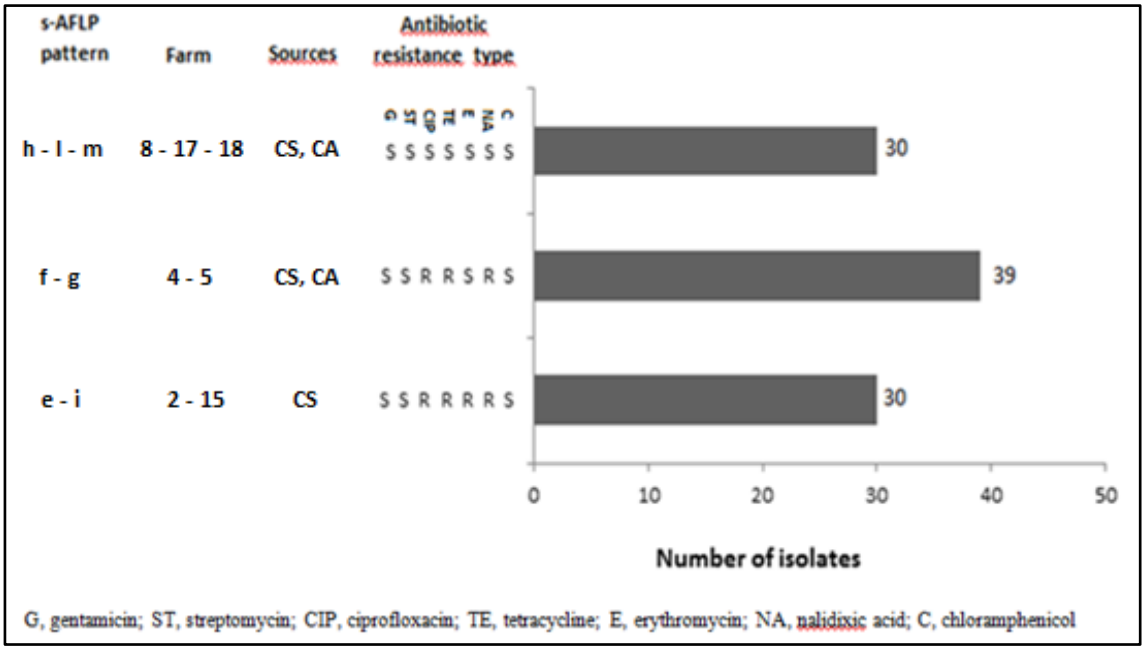


Figure 12. Antimicrobial resistance profiles and s-AFLP patterns of *C. coli* isolated from cloacal swabs (CS) on farm and carcass samples (CA) at the end of the processing line, after chilling

All *Campylobacter coli* strains isolated from farms 4 and 5 were co-resistant to two antimicrobials (quinolones and tetracycline) while all *C. coli* isolated from farms 2 and 15 displayed multi-resistances (quinolones, tetracycline and macrolides). In *Campylobacter jejuni* was revealed only co-resistance to quinolones and tetracycline in 4 strains isolated from farm 5 (Tab. 13).

RESISTANCE PATTERN PROFILE	**N° *C. coli* (%)**	**N° *C. jejuni* (%)**	**Farm**
QUIN, TET	39 (39)	4 (9.8)	4, 5
QUIN, TET, MAC	30 (30)	0 (0)	2, 15

QUIN: quinolones; TET: tetracyclines; MAC: macrolides

Table 13. Co-resistance and multi-resistance profiles of *C. coli* and *C. jejuni* (%)

2.3.4 Discussion

In this study, 50% broiler chickens farms were colonized with *Campylobacter*, while the contamination of carcasses was 36%. Of eleven positive broiler chicken farms, six farms showed colonization only in cloacal swabs, while the others five farms showed colonization both in cloacal swabs and neck skins. The cross-contamination of carcasses in slaughterhouse was observed only in three broiler chicken farms, which do not show *Campylobacter* colonization in cloacal swabs. In agreement with other studies (Aboaba and Smith, 2005; Padungtod and Kaneene, 2005; Huneau-Salaün *et al*., 2007; EFSA, 2010a), a high proportion of *C. coli*, isolated from broiler chicken farms and from processed broiler chicken carcasses, was reported. A study in Italian poultry production systems showed that 47.5% of isolates obtained from broiler chicken carcasses were *C. coli* (Manfreda *et al*., 2006).

In contrast to this result, *C. jejuni* was usually the dominant *Campylobacter* species isolated in broiler chickens (Hald *et al*., 2000; Cardinale et al., 2004; Wittwer *et al*., 2005; Näther *et al*., 2009; Sasaki *et al*., 2011); however the ratio of *C. coli* to *C. jejuni* varies between countries (Suzuki and Yamamoto 2009).

As expected (Rivoal *et al*., 2005; Wittwer *et al*., 2005; Denis *et al*., 2008; Giacomelli *et al*, 2012; Vidal *et al*., 2016), high levels of strain diversity among *C. coli* and *C. jejuni* isolates, in eight farms analyzed, were observed. The results showed the presence of a correlation between genotype and farm for *C. coli*, with a specific genotype pattern strictly correlated to each individual farm. Unlikely *C. jejuni*, thought fewer isolates were obtained, showed more diversity, in terms of number of genotypes for farm. A previous study already reported the presence of two or four different genotypes of *C. jejuni* in each individual bird in broiler farms (Kudirkiene *et al.* 2010).

Studies of monitoring of broiler chicken flocks have shown that some flocks may be infected by only one genotype of *Campylobacter* spp (Chuma *et al*., 1997; Hafez *et al*., 2001; Ring *et al*., 2005)

while other flocks may be infected by two or more different genotype of *Campylobacter* spp (Newell and Wagenaar, 2000; Hafez *et al*., 2001; Hiett *et al*., 2002; Denis *et al*., 2008); moreover, flocks may be colonized by a succession of different genotypes over time (Vidal *et al*., 2016).

A number of studies referred that genetic diversity was greater in *C. jejuni* than *C. coli*, responsible for a more stable population within a broiler flock compared to *C. jejuni* (Duim *et al.* 2001; Colles *et al.* 2015; Vidal *et al.* 2016). Furthermore the high diversity of *C. jejuni* is a possible consequence of the high frequency of genomic variation (Wittwer *et al*. 2005), or it could be due to the subsequent introduction of the bacterium (Bull *et al.* 2006 and Hue *et al*. 2011).

The same s-AFLP patterns of *C. coli* and *C. jejuni* were found in farms and slaughterhouse, while the novel patterns due to a possible cross contamination were not detected. It could be depend on the rational organization and biosecurity measures applied in the abattoir as reported by Sasaky et al. (2011). Several authors described the presence of the novel genotypes on the surface of the carcass likely caused by cross-contamination mostly occurred during post-defeathering and scalding processes (Corry *et al*. 2001; Johnsen *et al*. 2007; Ellerbroek *et al.,* 2010). The investigation on antimicrobial resistance showed a higher prevalence of *C.coli* resistant isolates (70%) to fluoroquinolone and tetracycline if compared to *C. jejuni* isolates (39% and 10% resistant isolates to fluoroquinolone and tetracycline respectively). This result could be related to the extensive use of fluoroquinolones and tetracyclines in food-producing animals in our area responsible for favoring the emergence of resistant *Campylobacter* isolates as reported by other studies (Aarestrup and Engberg, 2001; Ge *et al.* 2013). In agreement with other studies, we observed differences of antimicrobial resistance between *C. coli* and *C. jejuni*, with the latter being generally more susceptible (Jore *et al*. 2010; Haruna *et al*., 2012; Pollett *et al*. 2012; Ge *et al*. 2013; Wieczorek *et al*. 2013). Our results showed that 30% of *C. coli* isolates were resistant to erythromycin whereas all isolates of *C. jejuni* were susceptible. These data are in accordance with those reported by a recent study (Fraqueza *et al*., 2014) that indicated that several *Campylobacter* isolates from poultry reared in Portugal under different production systems displayed erythromycin resistance, often connected

with resistance to other antimicrobial classes. Similar results were described among *C. coli* isolates of poultry origin (23% of the erytromycin resistant strains) in Italy (Parisi *et al.* 2007). In contrast, *C. jejuni* appeared to be predominantly susceptible to erythromycin with low levels of resistance, detected in Japan (Haruna *et al*. 2012), in Italy (Pezzotti *et al*. 2003), in Lithuania (Ruzauskas *et al.* 2011) in Peru (Pollet *et al*. 2012). It should be emphasised that for clinical therapy of campylobacteriosis erythromycin is considered as the drug of choice (Xia *et al*. 2013). Although the incidence of resistance in human *Campylobacter* isolates is still relatively low, it could become higher, considering the fact that resistance to erythromycin has been increasing at the poultry production level as above mentioned. This event could be a consequence of an overuse of macrolides which are the most common therapeutics used in poultry farms because of the short or no withdrawal time. Multi-drug resistance (erythromycin, fluoroquinolones and tetracycline) was detected only in *C. coli* isolates and not in *C. jejuni* as reported by Wieczorek *et al*. (2013) and Di Giannatale *et al*. (2014).

The results showed that there is a significant correlation between genotype and antibiotic resistance profiles ($P < 0.001$), both for *C. jejuni* and for *C. coli* isolates, as observed by De Cesare *et al*. (2008). In this study *Campylobacter* isolates collected from each farm showed mostly the same genotype and the same antibiotic resistance profile. *C. jejuni* strains obtained from one same farm displayed two different genotypes with two antibiotic resistance profiles for each genotype as reported by other authors (Chu *et al.* 2004; Thakur and Gebreyes, 2005). In conclusion, genetic diversity was revealed among *Campylobacter* isolates in eight broiler farms analyzed. The higher levels of antibiotic resistance were found in *C. coli*; in particular the presence of high prevalence of erythromycin resistant isolates should be underlined raising a potential issue of public health.

CHAPTER 3. Study on *Campylobacter* contamination in raw broiler chicken meat products and effect of temperature and packaging on *Campylobacter* survival in raw chicken meats

3.1 Introduction

Poultry and poultry products have been implicated as a major source of *Campylobacter* infection in humans. There are few studies (Nobile *et al.*, 2013; Sammarco *et al.*, 2010; Parisi *et al.*, 2007; Pezzotti *et al.*, 2003) on the contamination of retail poultry meat in Italy and this studies were performed only in some regions. However, prevalence of *Campylobacter* in Italy ranged from 20.7 to 81.3% (Mezher *et al*., 2016).

The skin of broiler chickens is a major site of carcass contamination (Chantarapanont *et al*., 2003). *Campylobacter* can be transferred onto poultry carcasses via fluid and feces from broiler chickens' gastrointestinal tract, due to the high numbers of the organism found in these fluids (Franco and

Williams, 2001). The organism then can remain on the broiler chicken skin surface and perseveres to final products.

The majority of viable *Campylobacter* cells found on poultry skin have been located in crevices, entrapped in the surface water layer or entrapped with water in feather follicles (Chantarapanont *et al*., 2003). Different studies have shown that *C. jejuni* can survive on raw and cooked poultry samples during refrigeration at 4°C (Chantarapanont *et al*., 2003) and under frozen storage at -20°C (Lee *et al*., 1998).

The overuse of antibiotics on the broiler chicken farms poses a risk to human health, selecting drug-resistant bacteria that can be potentially transmitted to humans and may compromise clinical treatment (Chen *et al*., 2010).

The aim of this study was to evaluate the presence and the concentration of *Campylobacter* on broiler chicken meats with skin (thighs) and skinless (breasts), collected during processing at the time of packaging. Effect of refrigeration at 0°C and of packaging on survival of *Campylobacter* in broiler chicken meat samples at end shelf life was investigated.

Additionally, random isolated *C. jejuni* and *C. coli* strains were characterized for diversity genetic and for antimicrobial resistance and the presence of *Cdt* genes, associated with virulence, was investigated in *C. jejuni* strains, isolated from broiler chicken meat at end shelf life.

3.2 Material and Methods

3.2.1 Sampling

From October 2015 to July 2016 a total of 80 broiler chicken meat samples (40 breasts and 40 thighs) were randomly collected during poultry processing, within 1 h after slaughter and transported to the laboratory for microbiological analysis. Then, samples were repackaged in plastic film (PF) packaging or in modified atmosphere packaging (MAP), with 70% O_2, 20% CO_2, 10% N_2, and stored under refrigeration conditions (0°C) until the end of the shelf life. Then, these samples were transported to the laboratory for microbiological analysis.

3.2.2 Isolation and identification of *Campylobacter* spp

Enumeration of *Campylobacter* spp was performed according to the method described in ISO 10272-2:2006. The research of *Campylobacter* was made primarily from the skin material, if available, and secondly from meat, depending on the sample type e.g. skin and skinless poultry meat products. Twenty-five grams from each incised skin and chicken meat (thigh and breast) were aseptically put into a sterile bag and diluted using a ratio of 1:10 in Buffer Peptone Water (BPW). The mixture was then homogenized for 1 min in a peristaltic homogenizer. One mL of a 10^{-1} dilution was spread evenly over three mCCD agar plates (Oxoid). In addition, 0.1 mL of each 10^{-1}

dilution were inoculated and spread onto mCCD agar plates (Oxoid) and incubated in microaerophilic conditions (CampyGen, Oxoid) at 41.5 ± 0.5°C for 48 hours. Presumptive *Campylobacter* spp. colonies showing the typical morphology were counted. The limit for enumeration was 10 CFU/g.

For each positive plate, up to sixteen typical *Campylobacter* colonies were subcultured onto blood agar plates (Oxoid) and incubated under microaerophilic conditions (CampyGen, Oxoid) at 41.5 ± 0.5°C for 48 hours for further characterization according to the ISO method.
Isolates were confirmed by biochemical tests (oxidase test, catalase test, hippurate hydrolysis test), microscopic examination and gram staining. Colonies from Columbia blood agar were stored in Brucella broth (Biolife) supplemented with 30% glycerol at -80°C to await further analysis.

3.2.3 DNA extraction and molecular identification of *Campylobacter* genus and species

Strains phenotypically classified as *Campylobacter* were identified by conventional multiplex PCR assay, as previously described by Wang *et al* (2002). The strains used as positive controls were *C. coli* NCTC 11353, *C. fetus subsp.fetus* ATCC 27374, *C. jejuni* ATCC 33291, *C. upsaliensis* NCTC 11541 and *C. lari* NCTC 11552. Chromosomal DNA was extracted using an Ultraclean Microbial DNA Isolation kit (MO BIO Laboratories), quantified using a Nanodrop Spectrophotometer and DNA concentration was adjusted to approximately 50 ng/µl for each sample. The six pairs of primers were used to identify the genes *hipO* from *C. jejuni*; *glyA* from *C. coli*, *C. lari*, and *C. upsaliensis*; *sapB2* from *C. fetus* subsp. *fetus*; and the internal control 16S

rRNA. The PCR amplification was performed in a final volume of 50µl containing 5µL of 10X buffer, 200µM of each deoxynucleoside triphosphate, 1 U of *Taq* DNA polymerase, 0.5 µM of primers *C. jejuni* and *C. lari*; 1 µM of primers *C. coli* and *C. fetus*, 2 µM of primers *C. upsaliensis* and 5µL of DNA. The DNA extracted from the samples under analysis was used as template for amplification which provided an initial denaturation step at 95°C for 5 min followed by 30 cycles of amplification (denaturation at 95°C for 30 sec, annealing at 59°C for 30 sec, and extension at 72°C for 30 sec), with a final extension at 72°C for 7 min. The PCR products were detected on a 1.5% agarose gel, stained with 4 µL of ethidium bromide, run at 78 V for 40 min, visualized under UV light and photographed at the transilluminator.

The 16S PCR amplification was performed in a final volume of 50µl containing 5µL of 10X buffer, 200µM of each deoxynucleoside triphosphate, 1 U of *Taq* DNA polymerase, 1 µM of primers *Campylobacter* and 5µL of DNA. The amplification provided an initial denaturation step at 95°C for 5 min followed by 30 cycles of amplification (denaturation at 95°C for 30 sec, annealing at 52°C for 30 sec, and extension at 72°C for 30 sec), with a final extension at 72°C for 7 min. The PCR products were detected on a 1.5% agarose gel, stained with 4 µL of ethidium bromide, run at 78 V for 40 min, visualized under UV light and photographed at the transilluminator.

3.2.4 PFGE typing

Pulsed-field gel electrophoresis was conducted according to the instructions from the 2009 USA PulseNet protocol for *Campylobacter* (CDC, 2009).

Bacteria, previously identified by PCR as *C. jejuni* and *C. coli* were subcultured onto Columbia agar (Oxoid). Cell suspensions were prepared by removing the cells from the surface of the Columbia plates (Oxoid) using a cotton swab and suspending them in a small transparent tubes containing 2 ml of phosphate-buffered saline (PBS; 0.01 M phosphate buffer [pH 7.4], 0.85% NaCl). Each cell suspension was adjusted to an optical density of 0.35 to 0.45 using a Dade MicroScan turbidity meter; this corresponds to absorbance values of 0.570 to 0.820 at a wavelength of 610 nm when using a spectrophotometer. A 400-µl aliquot of adjusted cell suspensions was transferred to 1.5-ml microcentrifuge tubes containing 20 µl of proteinase K (20-mg/ml stock) and mixed gently with pipet tip. An equal volume (400 µl) of melted 1.0% SeaKem Gold (SKG) agarose in TE (10 mM Tris, 1 mM EDTA, pH 8.0) was added to the cell suspension, one sample at a time, and mixed gently by pipetting the mixture up and down two to three times. The agarose-cell suspension mixture was dispensed immediately into the wells of reusable plug molds. The agarose plugs were allowed to solidify at room temperature for 10 to 15 min or at 4°C for 5 min.

The plugs were transferred to 50 ml tubes (polypropylene tubes) containing 5 ml of cell lysis buffer (50 mM Tris, 50 mM EDTA [pH 8.0], 1% sarcosine, 0.1 mg of proteinase K/ml). Lysis was allowed to proceed for 15-30 min at 54-55°C in a shaker water bath with constant and vigorous agitation

(175-200 rpm). After lysis, the plugs were washed four times (15 to 20 min/wash) at 54-55°C (once with sterile ultrapure water and three times with TE, pH 8) in a shaking water bath. The water and TE were prewarmed at 54-55°C before each washing step. A 2-2,5 mm-wide slice from each plug was cut with a scalpel (or single edge razor blade, cover slip, etc.) and transferred to a tube containing 200 µl of diluited restriction buffer solution (1X). The plug slices were incubated in this restriction buffer at room temperature for 5 min. Then, the pre-restriction mixture was removed, and 200 µl of the restriction enzyme mixture containing 40 U of *Sma*I (Roche) was added to each tube. The plug slices were incubated at room temperature (25°C) for 2 h. *Salmonella* serovar *Branderup* H9812 was used as standard molecular weight size. Prior to casting of the gel, the restriction mixture was removed from each tube and replaced with 200 µl of 0.5× TBE (10X TBE contains 0.89 M Tris borate and 0.02 M EDTA, pH 8.3). The plug slices were allowed to stand at room temperature for 5 min, after which they were loaded into the appropriate wells of a 1% SKG agarose gel. The electrophoresis conditions consisted of an initial switch time of 6.8 s and a final switch time of 35.4 s (gradient of 6 V/cm and an included angle of 120°). These switch time values can be set using the AutoAlgorithim function of the CHEF Mapper (Bio-Rad) to separate fragments in the range of 50 to 475 kb. The gels were electrophoresed for 18-19 hours in 0.5X TBE.

After electrophoresis run, the gel was stained with Sybr Safe DNA gel stain and photographed at transilluminator. Restriction fragment migration profiles were compared by using the Bionumerics program v. 6.6 (Applied Maths NV, Sint-Martens-Latem, Belgium).

3.2.5 Antimicrobial Susceptibility testing

Campylobacter isolates susceptibilities to antibiotics were evaluated by MIC. Colonies were picked and grown on Columbia agar (Oxoid) at 42°C in a microaerophilic atmosphere for 24 h. Next, the colonies were seeded in Mueller Hinton Broth supplemented with blood and dispensed into Eucamp microtitre plates containing known scalar concentrations of the following antibiotics: gentamicin (0.12–16 μg/ml), streptomycin (1–16 μg ml^{-1}), ciprofloxacin (0.06–4 μg ml^{-1}), tetracycline (0.25–16 μg $ml^{-1)}$, erythromycin (0.5–32 μg ml^{-1}), nalidixic acid (2–64 μg ml^{-1}) and chloramphenicol (2–32 μg ml^{-1}). After inoculation, the plates were incubated at 42°C in a microaerophilic atmosphere for 24 h and then screened. *C. jejuni* strain NCTC 11351 was used as the control. The results for chloramphenicol were evaluated according to the interpretation criteria of the French Society of Microbiology, while breakpoints established from EUCAST for *Campylobacter* spp. were used for all other antibiotics.

3.2.6 Multiplex PCR to detect the presence of CDT toxin genes in *C. jejuni* strains

Multiplex PCR assay, described by Asakura *et al*. (2008), was used to detect the simultaneous presence of *cdt*A, *cdt*B, *cdt*C genes virulence in the strains of *Campylobacter jejuni*, previously isolated from broiler chicken meat at end of shelf life.

The three pairs of primers were used to identify the genes *cdt*A, *cdt*B, *cdt*C from *C.jejuni*. The PCR amplification was performed in a final volume of 50µl containing 5µL of 10X buffer, 200µM of each deoxynucleoside triphosphate, 1 U of *Taq* DNA polymerase, 20 pmol of each primer (*CjspAU2, CjspAR2, CjSPBU5, CjSPBR6, CjspCU1* and *CjspCR2*) and 5µL of DNA. The amplification cycle provided an initial denaturation step at 94°C for 5 min followed by 30 cycles of amplification (denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec), with a final extension at 72°C for 10 min. The PCR products were detected on a 1.5% agarose gel, stained with 4 µL of ethidium bromide, run at 78 V for 40 min, visualized under UV light and photographed at the transilluminator.

The *cdtA* gene corresponds to a fragment of 631 bp; *cdtB,* to a fragment of 714 bp; and *cdtC,* to a fragment of 524 bp.

Primer	Sequence (5'-3')	Target gene	Microorganism
C*jspAU2* C*jspAR2*	5'-AGGACTTGAACCTACTTTTC-3' 5'-AGGTGGAGTAGTTAAAAACC-3'	*cdt*A	*C. jejuni*
C*jSPBU5* C*jSPBR6*	5'-ATCTTTTAACCTTGCTTTTGC-3' 5'-GCAAGCATTAAAATCGCAGC-3'	*cdt*B	*C. jejuni*
C*jspCU1* C*jspCR2*	5'-TTTAGCCTTTGCAACTCCTA-3' 5'-AAGGGGTAGCAGCTGTTAA-3'	*cdt*C	*C. jejuni*

Table 14. Primer used for multiplex PCR

3.2.7 Statistical analysis

Statistical analysis software Bionumerics program v. 6.6 (Applied Maths NV, Sint-Martens-Latem, Belgium) was used to obtain an estimation of *Campylobacter jejuni* and *Campylobacter coli* populations in broiler chicken meats and to represent graphically the distance among the resulting pulsotypes, performing the image analysis. The similarity analysis was carried out using the Dice coefficient (position tolerance, 1%). The unweighted pair group mathematical average was used to cluster patterns. Isolates with <90% similarity were clustered as separate pulsotypes.

3.3 Results

A total of 80 broiler chicken samples (40 thighs and 40 breast) was collected during poultry processing, within 1 h after slaughter, to determine *Campylobacter* contamination in broiler chicken meat products. Each samples were repackaged in two different packaging (plastic film (PF) packaging and modified atmospheric packaging (MAP)) and storage at refrigeration temperature (0°C) until end shelf life to investigate the survival of *Campylobacter* spp.

Genetic diversity and antimicrobial resistance of random *Campylobacter jejuni* and *Campylobacter coli* strains was investigated. Furthermore, the presence of *cdt* virulence genes was examined in *C. jejuni* strains isolated from broiler chicken meats at end shelf life.

3.3.1 Presence of *Campylobacter* in broiler chicken meat products

3.3.1.1 Isolation and identification

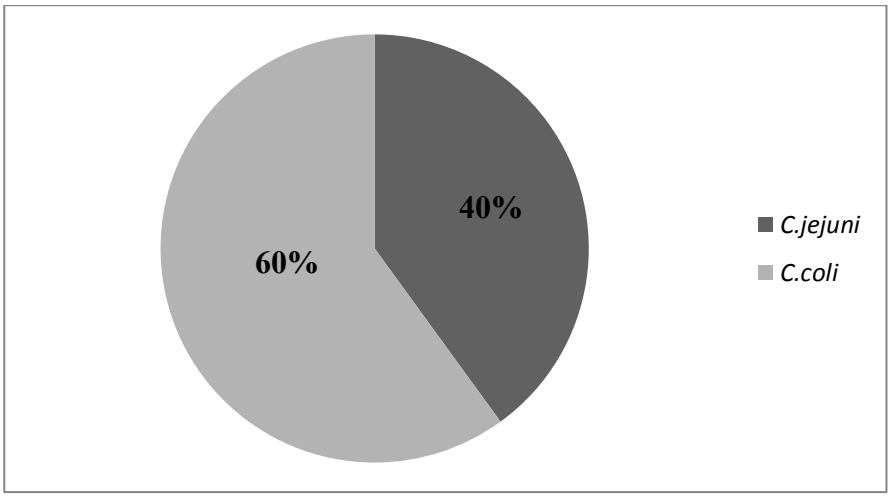
From 80 broiler chicken meat samples analyzed, 52 (65%) were positive for *Campylobacter* spp and 28 (35%) were free of *Campylobacter* spp contamination.

As shown in Table 14, products with skin were more heavily contaminated than products without skin, with a presence of *Campylobacter* of 85.0% (34/40) on the thighs and 45.0% (18/40) on the breasts. The species found in the 242 isolates collected from positive samples were determined by multiplex PCR and revealed that *C. jejuni* was isolated 68% from chicken meat products while

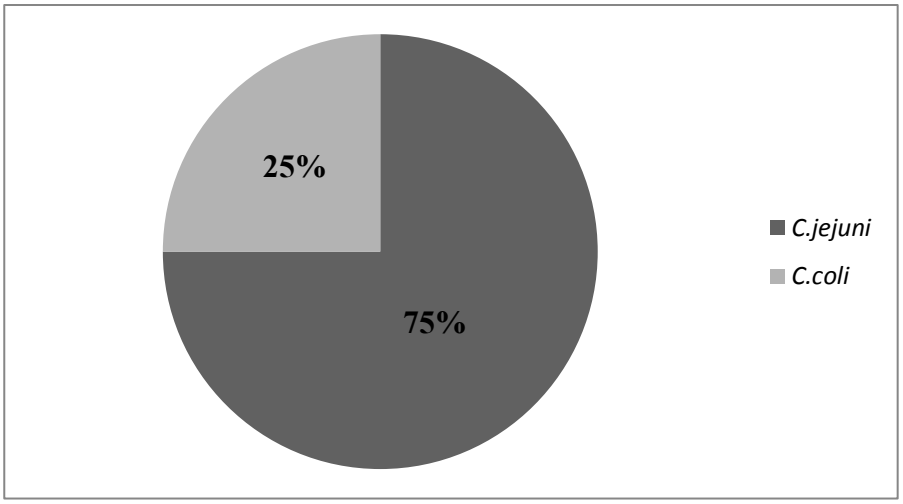
C. coli was isolated 32%. Thus, *C. jejuni* was more prevalent than *C. coli* in fresh broiler chicken meat products (Tab 15).

Fresh meat	**No of positive samples/ No of all samples (positive %)**	**Number of *C. jejuni* strains**	**Number of *C. coli* strains**	**Total *Campylobacter* strains**
Breasts (without skin)	18/40 (45.0)	19	28	47
Thighs (with skin)	34/40 (85.0)	146	49	195
Total	**52/80 (65.0)**	**165**	**77**	**242**

Table 15. *Campylobacter* in two different cuts of broiler chicken meats



Graphic 3. Proportions of *C. jejuni* and *C. coli* identified among *Campylobacter* spp strains (n=47), isolated from broiler chicken breast samples



Graphic 4. Proportions of *C. jejuni* and *C. coli* identified among *Campylobacter* spp strains (n=195), isolated from broiler chicken thighs samples

Of the 18 positive broiler chicken breast samples analyzed in this study, 4 had only the *C. jejuni* species, 6 had only *C. coli*, and 6 were contaminated by both species (Tab. 16); while of the 34 positive broiler chicken thighs samples, 19 had only the *C. jejuni* species, 3 had only *C. coli*, and 12 were contaminated by both species (Tab. 17).

Positive Breast samples	**Number of samples with *C.jejuni* only**	**Number of samples with *C.coli* only**	**Number of samples with *C.jejuni* and *C.coli***	**Number of samples with *Campylobacter* spp**
18	4	6	6	2

Table 16. *Campylobacter* species in broiler chicken breast samples

Positive Thighs samples	**Number of samples with *C.jejuni* only**	**Number of samples with *C.coli* only**	**Number of samples with *C.jejuni* and *C.coli***	**Number of samples with *Campylobacter* spp**
34	19	3	12	0

Table 17. *Campylobacter* species in broiler chicken thighs samples

The results of the *Campylobacter* enumeration on fresh broiler chicken meats in this study were categorized as follows: <100 CFU/g; 100-499 CFU/g; 500-1000 CFU/g and >1000 CFU/g (Tab.18). Enumeration of *Campylobacter* in breast samples varied from a minimum of 10 CFU/g to a maximum of 295 CFU/g while in thigh samples the enumeration of *Campylobacter* varied from a minimum of 10 CFU/g to a maximum of 8300 CFU/g .

Fresh meat	***Campylobacter* counts (CFU/g)**			
	<100	**100 - 499**	**500 - 1000**	**>1000**
Breasts	14 (78)	4 (22)	0	0
Thighs	13 (38)	15 (44)	2 (6)	4 (12)

Number of sample (percentage)

Table 18. *Campylobacter* enumeration data obtained from fresh broiler chicken meats

There was a reduction of positive samples (42%, 22/52), after the refrigeration at 0°C until end shelf life, with higher reduction found in broiler chicken breast samples than broiler chicken thighs samples. Multiplex PCR revealed that *C. jejuni* was isolated 72% from chicken meat products while *C. coli* was isolated 28%. Thus, also in this case, *C. jejuni* was more prevalent than *C. coli* in broiler chicken meat products at end shelf life (Tab. 19).

Fresh meat	**No of positive samples at end shelf-life after refrigeration at 0°C**	**Number of *C. jejuni* strains**	**Number of *C. coli* strains**	**Total *Campylobacter* strains**
Breast (without skin)	1/18 (5.5)	0	1	1
Thighs (with skin)	21/34 (68.0)	52	19	71
Total	**22/52 (42)**	**52**	**20**	**72**

Table 19. *Campylobacter* in two different cuts of broiler chicken meats at end shelf life

Of the 21 positive broiler chicken thighs samples analyzed at end shelf life in this study, 15 had only the *C. jejuni* species, 1 had only *C. coli*, and 5 were contaminated by both species (Tab. 20), while the only positive sample of breast analyzed at end shelf life was contaminated by only *C.coli* (Tab. 19).

Positive Thighs samples at end shelf life	**Number of samples with *C.jejuni* only**	**Number of samples with *C.coli* only**	**Number of samples with *C.jejuni* and *C.coli***	**Number of samples with *Campylobacter* spp**
21	15	1	5	0

Table 20. *Campylobacter* species in broiler chicken thighs samples at end shelf life

In this study, the influence of MAP and PF packaging on reduction of *Campylobacter* growth rate in broiler chicken meats at end shelf life was observed.

Campylobacter counts were reduced in both packaging used in this study (Tab. 21).

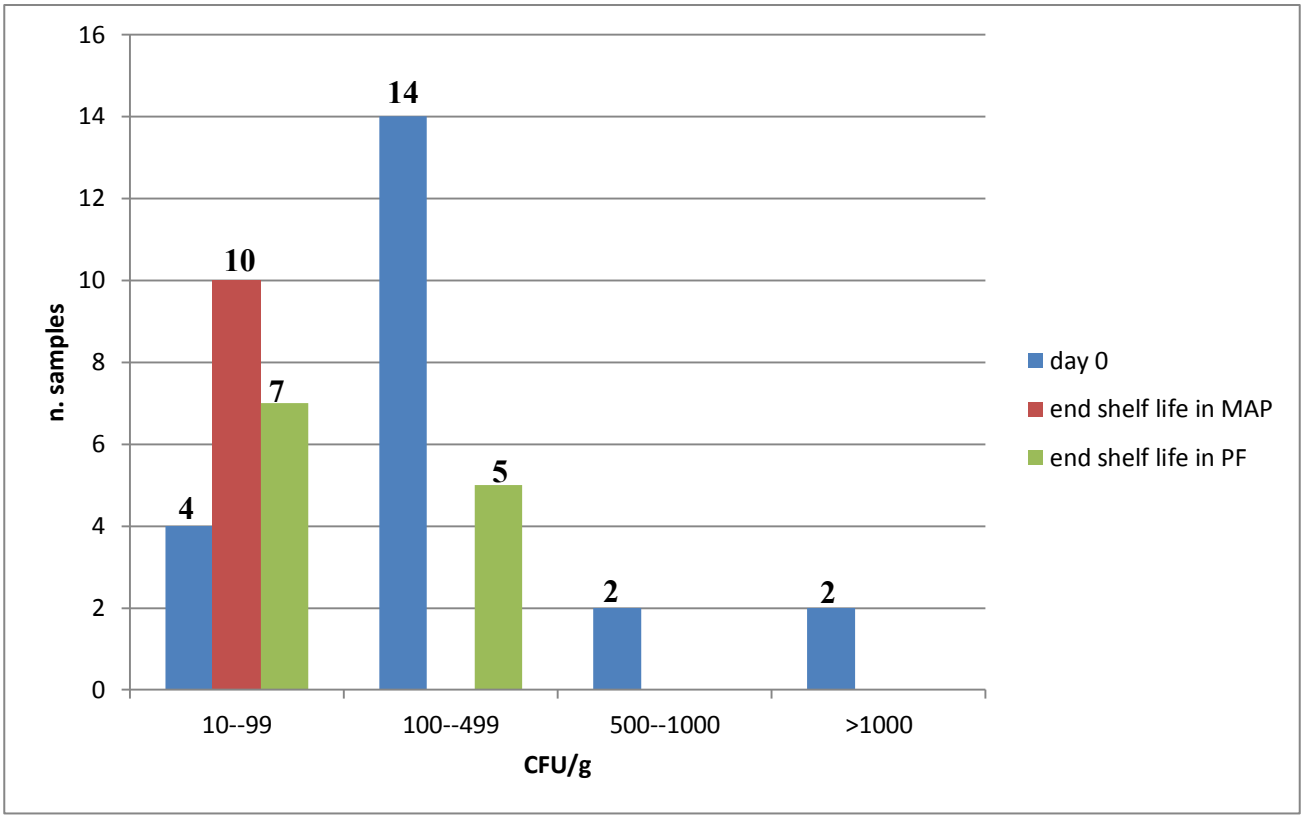


Table 21. *Campylobacter* enumeration data obtained from broiler chicken meats at end shelf life

Particularly, *Campylobacter* counts (in this case expressed in $\log_{10}$) were reduced mostly in chicken thigh samples packaged in MAP (Tab. 22). A reduction of 1.69 $\log_{10}$ to 3.91 $\log_{10,}$ with average value of 2.48±0.73, was observed in viable *Campylobacter* counts after chilling storage of thigh samples packaged in MAP.

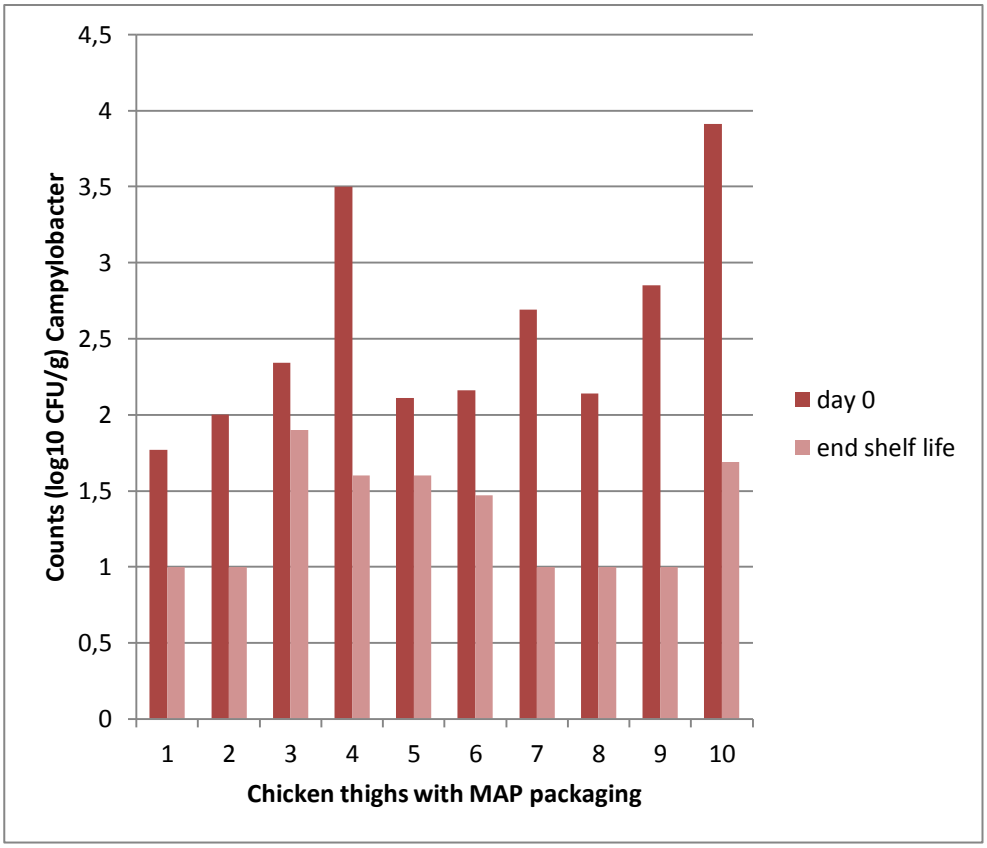


Table 22. Reduction *Campylobacter* counts in chicken thighs packaged in MAP

Campylobacter counts were reduced less in chicken thigh samples packaged in plastic film (PF) (Tab. 23). A reduction of 1.30 $\log_{10}$ to 2.71 $\log_{10,}$ with average value of 1.53±0.89, was observed in viable *Campylobacter* counts after chilling storage of thigh samples packaged in PF. In only positive sample of breast, viable *Campylobacter* counts do not reduce (Tab. 24).

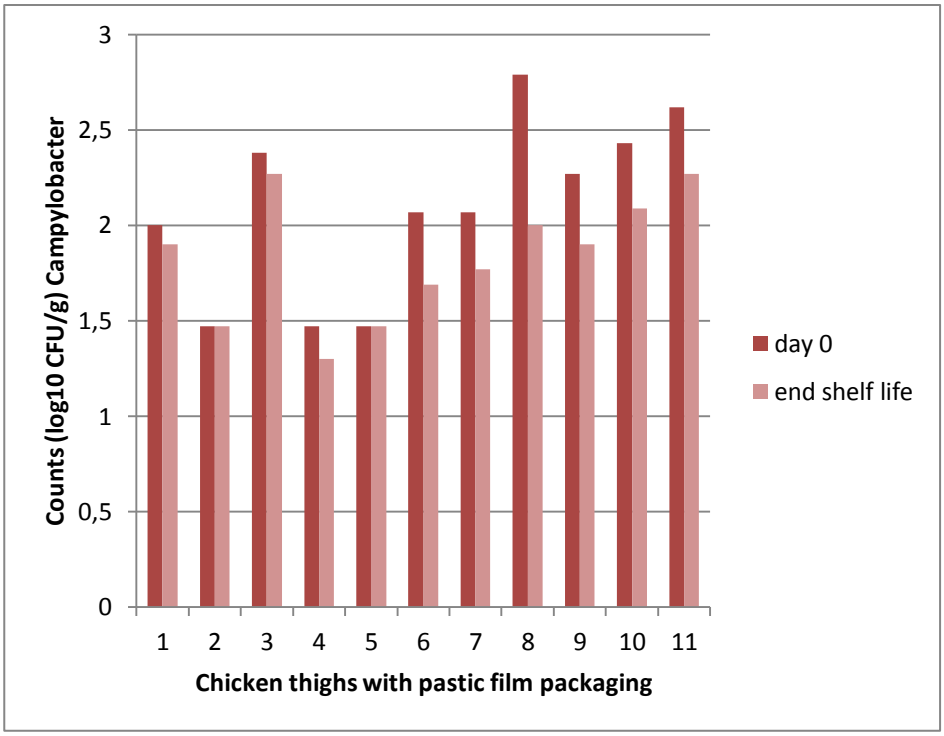


Table 23. Reduction *Campylobacter* counts in chicken thighs packaged in PF

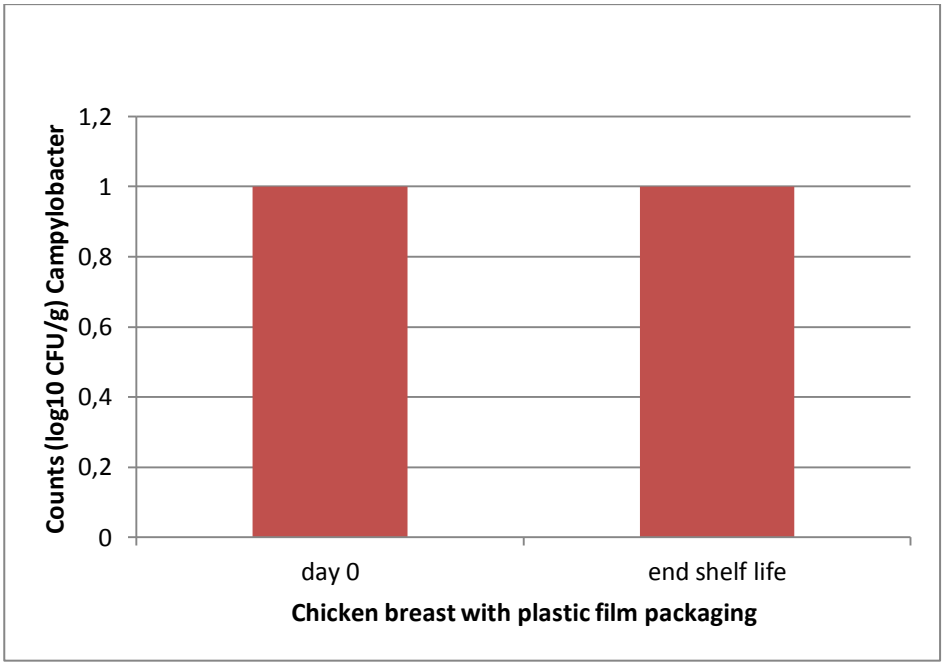


Table 24. *Campylobacter* counts in chicken breast packaged in PF

3.3.2 PFGE typing

A total of 23 random *Campylobacter jejuni* strains and 9 random *Campylobacter coli* strains (Tab. 25 - 26) isolated from fresh broiler chicken meats at day of packaging (day 0) and from broiler chicken meat at end shelf life were characterized by pulsed-field gel electrophoresis (PFGE) using *Sma*I restriction enzyme and by antimicrobial susceptibility testing.

Souces	**N° samples at day 0**	**N° *C. jejuni* strains**	**N° *C. coli* strains**
Thighs	16	13	4

Table 25. *C. jejuni* and *C. coli* isolates recovered from broiler chicken meats at day of packaging

Souces	**N° samples at end shelf life**	**N° *C. jejuni* strains**	**N° *C. coli* strains**
Thighs	10	10	5

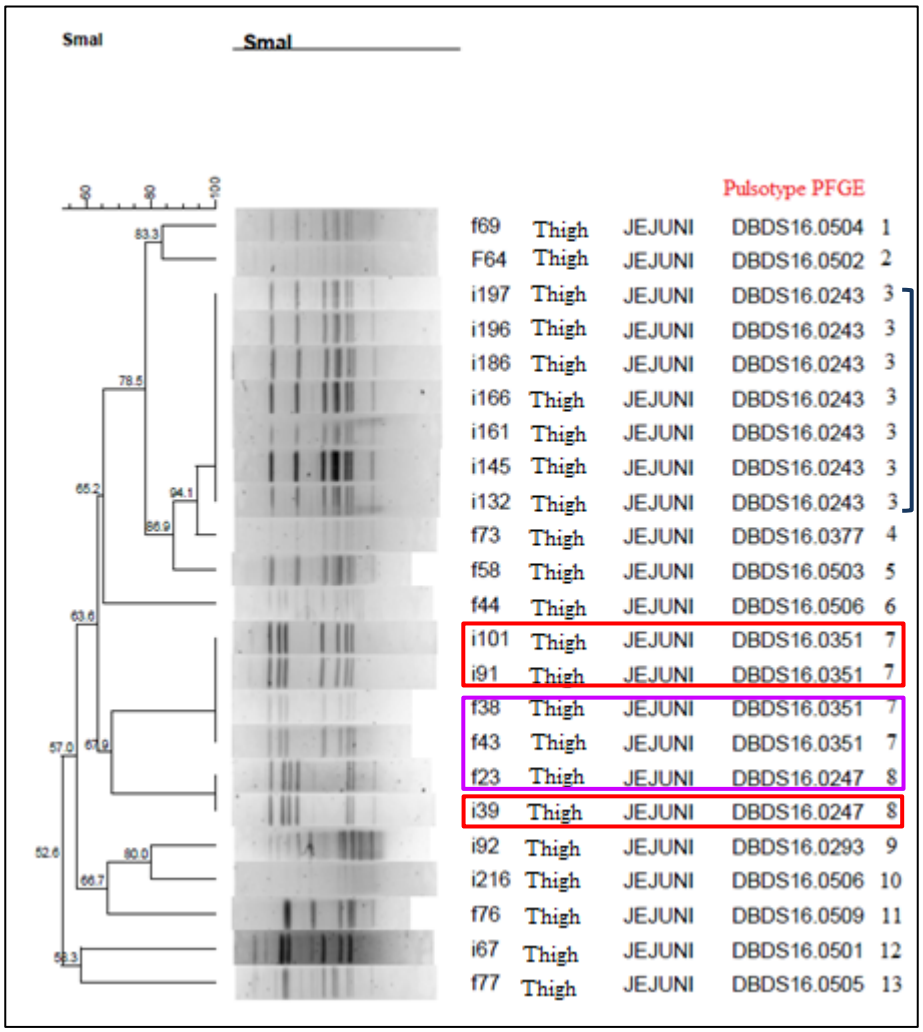
Table 26. *C. jejuni* and *C. coli* isolates recovered from broiler chicken meats at end shelf life

Thirteen different pulsotypes were obtained from *C.jeuni* strains typed (Fig.13).

Particularly, *C.jejuni* strains isolated from fresh broiler chicken thigh samples were characterized by 6 different pulsotype, with one pulsotype predominant (P3). The same pulsotype (P3) was recovered from 6 fresh broiler chicken thigh samples analyzed in different days (Fig. 13).

From the analysis of the *C. jejuni* strains isolated from broiler chicken thigh samples at end shelf life, 3 showed the same pulsotypes (P7-P8) found in fresh broiler chicken meat samples.

The remaining *C. jejuni* strains isolated from broiler chicken meat samples at end of shelf life showed 7 different pulsotypes (Fig.13).



i = initial (day 0);

f = final (end shelf life)

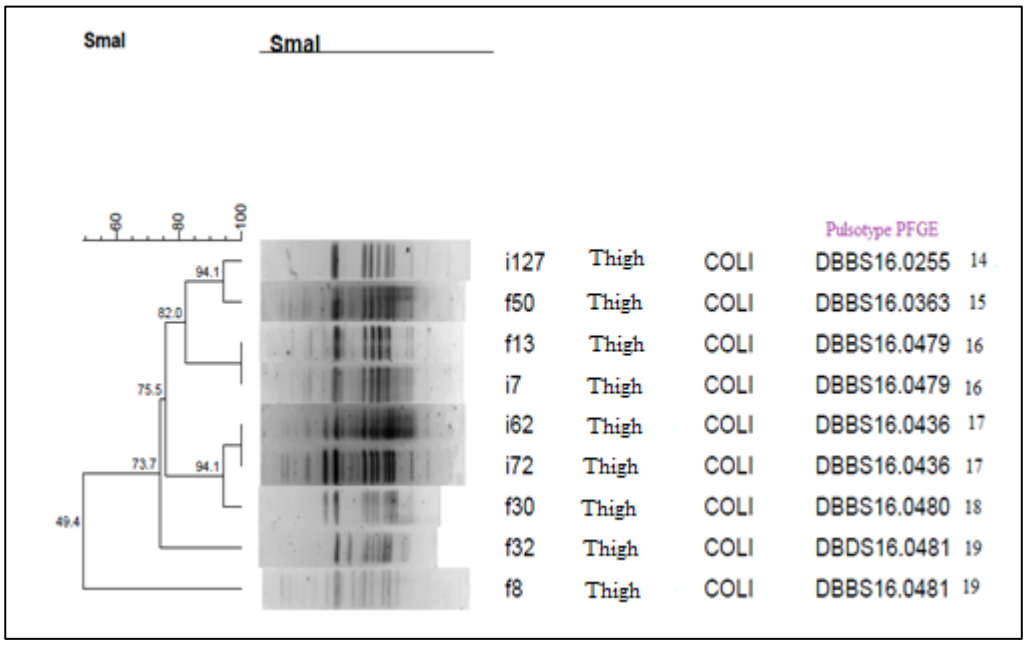
Pulsotype	Broiler chicken meat at day 0	Broiler chicken meat at end shelf life
	P3-P7-P8-P9-P10-P12	P1-P2-P4-P5-P6-P7-P8-P11-P13

Figure 13. Dendrogram of *C. jejuni Sma*I PFGE patterns isolated from broiler chicken meats

Six different pulsotypes were obtained from *C. coli* strains typed (Fig.14).

Particularly, *C. coli* strains isolated from fresh broiler chicken thighs samples were characterized by 3 different pulsotype, with one pulsotype (P17) found in two different thigh samples (Fig.14). From the analysis of the *C. coli* strains isolated from broiler chicken thigh samples at end shelf life, one showed the same pulsotypes (P16) found in fresh broiler chicken meat samples.

The remaining *C. coli* strains isolated from broiler chicken meat samples at end shelf life showed 3 different pulsotypes (Fig 14).



i = initial (day 0);

f = final (end shelf life)

Pulsotype	Broiler chicken meat at day 0	Broiler chicken meat at end shelf life
	P14-P16-P17	P15-P16-P18-P19

Figure 14. Dendrogram of *C. coli Sma*I PFGE patterns isolated from broiler chicken meats

3.3.3 Antimicrobial Susceptibility testing

Higher rates of resistances were observed in *Campylobacter jejuni* and *Campylobacter coli* for ciprofloxacin, tetracycline, erythromycin and nalidixic acid (Tab 27 – Tab. 28).

N° *C.jejuni* strains (%)						
	GM	**S**	**CIP**	**TE**	**E**	**NA**
R	0	1 (4)	23 (100)	21 (91)	8 (35)	21 (91)
I	0	7 (30)	0	0	15 (65)	0
S	23 (100)	15 (65)	0	2 (9)	0	2 (9)

Table 27. Antibiotic resistance profiles of *C. jejuni* isolates

N° *C.coli* strains (%)						
	GM	**S**	**CIP**	**TE**	**E**	**NA**
R	1 (11)	0 (4)	7 (78)	6 (67)	3 (33)	6 (67)
I	0	4 (44)	0	0	6 (67)	0
S	8 (89)	5 (56)	2 (22)	3 (33)	0	3 (33)

Table 28. Antibiotic resistance profiles of *C. coli* isolates

Multiple resistance was common in *C. jejuni*, with three patterns being recognized: strains were resistant for ciprofloxacin, nalidixic acid and tetracycline, or for ciprofloxacin, nalidixic acid, tetracycline and erythromycin or for ciprofloxacin, nalidixic acid, tetracycline and aminoglycosides. Single resistances were not observed (Tab. 27 - 29).

For *C. coli*, combined resistance for ciprofloxacin, nalidixic acid and tetracycline and multiple resistance for ciprofloxacin, nalidixic acid, tetracycline and erythromycin or for ciprofloxacin,

nalidixic acid, tetracycline, erythromycin and aminoglycosides were revealed to a lesser extent. Single resistances for tetracycline were observed only in two *C.coli* strains (Tab. 28 - 29).

RESISTANCE PATTERN PROFILE	**N° *C. jejuni* strains (%) isolated at day 0**	**N° *C. jejuni* strains (%) isolated at end of shelf life**	**N° *C. coli* strains (%) isolated at day 0**	**N° *C. coli* strains (%) isolated at end of shelf life**	**Total *C.jejuni* (%)**	**Total *C.coli* (%)**
QUIN, TET	7 (54)	5 (50)	0 (0)	1 (20)	12 (52)	1 (11)
QUIN, TET, MAC	5 (38)	3 (30)	0 (0)	2 (40)	8 (35)	2 (22)
QUIN, TET, AMIN	1 (8)	0 (0)	0 (0)	0 (0)	1 (4)	0 (0)
QUIN, TET, MAC, AMIN	0 (0)	0 (0)	1 (11)	0 (0)	0 (0)	1 (11)

Table 29. Co-resistance and multi-resistance profiles of *C. jejuni* and *C. coli* (%)

3.3.4 Detection of CDT toxin genes in *C. jejuni* strains

The presence of the virulence *cdt* genes was investigated in all 52 *C. jejuni* strains recovered from broiler chicken thighs at end shelf life (Tab. 30).

The *Cdt* genes were detected in 77% (40/52) of the *C. jejuni* strains isolates from broiler chicken thighs at the end shelf life. The *cdt* gene complex was not found in 12/52 (23%) *C. jejuni* strains.

Genes *cdt*A, *cdt*B and *cdt*C were simultaneously detected in 15/40 (37%) *C. jejuni* strains.

In 9/40 (22%) *C. jejuni* strains, genes *cdt*A and *cdt*C were found in broiler chicken thighs. In 10/40 (25%) strains, genes *cdt*A and *cdt*B were found in broiler chicken thighs. Furthermore, in 6/40 (15%) strains, only gene *cdt*C was detected (Tab. 30).

C.jejuni Strains	*cdt*A	*cdt*B	*cdt*C
f6	+	+	+
f9	+	-	+
f10	-	-	+
f11	-	-	-
f12	+	+	+
f14	-	-	-
f15	+	+	+
f16	-	-	+
f17	+	-	+
f18	+	+	+
f19	+	+	+
f20	+	+	+
f21	+	+	+
f22	+	+	+
f23	+	+	+
f24	+	+	+
f25	+	+	+
f26	+	+	+
f38	-	-	-
f39	+	-	+
f40	+	-	+
f41	-	-	+
f42	+	-	+
f43	+	+	+
f44	+	-	+
f45	+	+	+

f47	-	-	+
f49	+	-	+
f52	-	-	+
f53	+	+	+
f54	-	-	+
f55	-	-	-
f56	+	+	-
f57	+	-	+
f58	+	-	+
f59	-	-	-
f60	-	-	-
f61	-	-	-
f64	-	-	-
f68	+	+	-
f69	+	+	-
f70	-	-	-
f71	+	+	-
f72	+	+	-
f73	+	+	-
f74	+	+	-
f76	+	+	-
f77	+	+	-
f78	-	-	-
f79	-	-	-
f80	-	-	-
f81	+	+	-

Table 30. Detection of the *cdt* gene complex in *C. jejuni* strains isolated from broiler chickens thighs at end shelf life

Therefore, 4 different virulence genes *cdt* profiles was observed in *C. jejuni* strains (Fig.15).

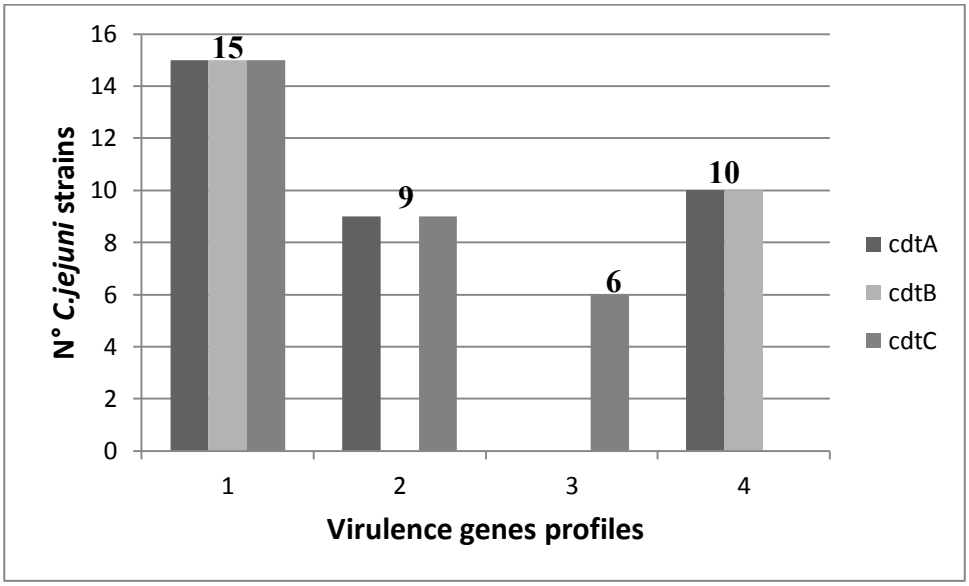


Figure 15. Virulence genes profiles in strains of *C. jejuni* isolated from broiler chicken thighs at end shelf life

3.3.5 Discussion

Studies indicated that retail broiler chicken meats *Campylobacter* are often contaminated with *Campylobacter* (Shih, 2000; Nobile *et al.*, 2013; Sammarco *et al.*, 2010; Parisi *et al.*, 2007; Pezzotti *et al.*, 2003). The results obtained reports that the presence of *Campylobacter* in fresh broiler chicken products is high; 65% of products analyzed resulted to be contaminated. Furthermore, this study showed that broiler chicken meat products with skin, such as the broiler chicken thighs, are the most heavily contaminated than broiler chicken products without skin, such as breast fillets. Indeed, low incidence of *Campylobacter* on skinless poultry products was found, suggesting that *Campylobacter* may not survive on skinless poultry meat. Several studies (Scherer *et al*. 2006, Luber and Bartelt 2007) underlined that the skin surface of carcasses is the part more contaminated of broiler chicken meat products, suggesting that cross-contamination from raw broiler chicken meat surface to ready to eat foods is high risk of *Campylobacter* infection.

Broiler chickens skin provides suitable microenvironment for the survival of *Campylobacter* and skin swelling due to water increases the surface area available for contamination by bacteria (Chantarapanont *et al*., 2003). More than one species of *Campylobacter* (*C. jejuni* and *C. coli*) was identified in broiler chicken meat samples analyzed. In agreements with other studies (Pezzotti *et al.,* 2003; Sammarco *et al.,* 2010; Guyard-Nicodème *et al*., 2015) *C. jejuni* was the dominant species isolated from broiler chicken meat products. Lower *Campylobacter* contamination counts (< 1000 CFU/g) in fresh broiler chicken products were found; only 8% of fresh broiler chicken meat products (thighs) showed a load higher (>1000 CFU/g). These high counts of *Campylobacter* were associated with the thigh skins contamination. In agreements with other studies (Lee *et al*., 1998; Chan *et al*., 2001; Solow *et al*., 2003; Haddad *et al*., 2009; Maziero *et al*., 2010; Awadallah *et al*., 2014), a reduction of *Campylobacter* contamination were seen in broiler chicken meat samples after refrigeration storage.

A decline of 2.48±0.73 CFU/g was observed in broiler chicken meat samples at end shelf life after refrigeration storage in modified atmosphere packaging (MAP), in agreement with Meredith *et al.*, (2014) and Boysen *et al*., (2006), and of 1.53±0.89 CFU/g after refrigeration storage in plastic film (PF) packaging. *Campylobacter* counts decreases higher in MAP packaging than PF packaging. Since *Campylobacter* is an oxygen-sensitive microaerophile, the presence of O_2 in the MAP gaseous mixtures, in ratio 70:20:10% O_2:CO_2:N_2, induced a decrease in *Campylobacter* levels. Furthermore this study showed that *C. jejuni* strains survival, following refrigeration storage, was greater than *C. coli* strains. Several studies have shown that *C. jejuni* strains can survive on raw or cooked broiler chicken meat samples during refrigeration (Lee *et al*., 1998; Chantarapanont *et al*., 2003; Solow *et al*., 2003).

Our study revealed that refrigeration could lethally injure and reduce cell counts of *Campylobacter* in broiler chicken meat samples at end shelf life after refrigeration storage in two different packaging (Maziero *et al*., 2010). In this form such bacteria cannot be cultivated on conventional media, but their existence can be provide using other methods, such as the detection of gene expression by reverse transcription polymerase chain reaction (RT-PCR) (Li *et al*., 2014).

Macrolides and fluoroquinolones are regarded as drugs of choice for the treatment of human *Campylobacter* infections. The possible use of antimicrobials for prophylactic and therapeutic purposes in broiler chickens induced the resistance of *Campylobacter* spp. to selected antibiotics, leading the emergence of fluoroquinolone-macrolide resistance isolates. This multidrug resistance caused severe issues in the treatment of resistance *Campylobacter* infections (Luangtongkum *et al*., 2009). Chai *et al*. (2008a) suggested that *C. jejuni* resistance towards fluoroquinolone is associated to farming practices. Since campylobacteriosis is transmitted primarily through consumption of food of animal origin, the presence of antimicrobial-resistant *Campylobacter* in raw poultry meat products has important public health implications. In addition, *Campylobacter* antimicrobial-resistant strains can be more virulent than

Campylobacter susceptible strains, causing more prolonged or more severe illness than antimicrobial-susceptible strains (Travers and Barza, 2002) .

Campylobacter jejuni isolates displayed high prevalence of resistance to ciprofloxacin, tetracycline, erythromycin and nalidixic acid.

All *C. jejuni* and *C. coli* strains isolated from broiler chicken meat sample were resistant to at least one of the six antimicrobials tested. Furthermore, in agreement with Ge *et al*., 2006, resistance to two or more classes of antibiotics was found in the majority of *Campylobacter* spp (78%) and among them one *C. coli* strain showed resistance to four different classes of antimicrobials.

In this study, PFGE was used to determine the genomic diversity of random *C. jejuni* and *C. coli* isolates recovered from broiler chicken meat samples at day of packaging and at end shelf life.

Typing of *C. jejuni* and *C. coli* by PFGE generated 13 and 6 patterns, respectively, indicating a diverse population of *C. jejuni* and *C. coli* present in broiler chicken meats, in agreement with other studies (Ge *et al*., 2006; Zorman *et al*., 2006, Praakle-Amin *et al.*, 2007). The common pulsotype found in *C. jejuni* strains isolated from fresh broiler chicken meat samples was P3. Interestingly, the presence in this study of one and two pulsotypes of *C. coli* and *C. jejuni* recovered both from fresh broiler chicken meat samples and from broiler chicken meat samples at end shelf life, stored in refrigeration temperature, suggested that specific genotypes of *Campylobacter* can survive under unfavorable conditions. Instead some genotypes were not recovered from broiler chicken meats after chilling storage despite being present in fresh broiler chicken meats. This suggests that there is a considerable differential in the ability of *Campylobacter* strains to survive environmental stresses. *Campylobacter jejuni* displays high genetic diversity and high events of intra-genomic recombination enhancing the its survival under different adverse conditions (Bolton, 2015). The ability of *Campylobacter* to survive to environmental stresses can depend on the presence and expression of stress (Newell *et al*., 2001) and virulence genes (González-Hein *et al*., 2014).

However, the storage at refrigeration temperature prevents deterioration of the broiler chicken meat but some poultry meat samples can show bacterial survivors after refrigeration.

Virulence markers analyzed in this study were the *cdt* genes. The result of CDT activity can differ depending on the type of eukaryotic cell affected.

The activity of the toxin CDT occurs by blocking the G2/M phase of eukaryotic cells prior to cell division, induces a cytoplasmic distention and finally causes cell death (Martinez *et al*., 2006).

CDT contributes to pathogenesis by inhibiting both cellular and humoral immunity via apoptosis of immune response cells, and also by generating necrosis of epithelial cells and fibroblasts involved in the repair of lesions produced by *Campylobacter*, resulting in slow healing and production of disease symptoms (Smith and Bayles, 2006).

Cdt genes were detected in 77% (40/52) of the *C. jejuni* strains isolates from broiler chicken meats at the end shelf life. The presence and expression of the three genes *cdt*A, *cdt*B, *cdt*C is required for CDT activity (Jeon *et al.*, 2005). Therefore, 37% (15/40) of the 40 strains that carried the *cdt* genes, were potentially virulent, once they had *cdtA, cdtB* and *cdtC*, simultaneously. CDT production of *C. jejuni* is associated with strains that cause gastroenteritis in humans (Van Deun *et al*., 2007).

Interestingly, two specific genotypes of *C. jejuni*, that survive at refrigeration conditions until end shelf life, showed all three *cdt* genes, in agreement with González-Hein *et al*., 2014, and resistance to fluoroquinolone and tetracycline.

However, the presence of *C. jejuni* strains potentially virulent for human is a possible public health risk.

CHAPTER 4. Conclusions

This study has allowed to investigated the presence and quantification of *Campylobacter* contamination along production chain of broiler chickens. Moreover survival *Campylobacter* in refrigeration storage in two different packaging, antibiotic resistance, diversity genetic and virulent genes were investigated.

A wide colonization with *C. jejuni* and *C. coli* of broiler chickens, both in farms, located in central Italy, and in poultry meat products was observed. A lower presence was revealed in chilled carcasses after processing line. The presence of both species of *Campylobacter* was observed, with different prevalence. Indeed, *Campylobacter coli* was the dominant species isolated in cloacal swabs of broiler chickens on farms and in neck skins of chilled carcasses at slaughterhouse while a greater presence of *C. jejuni* was revealed from broiler chicken meat samples, both at day of packaging and end shelf life. Therefore, monitoring *Campylobacter* presence in broiler chickens on farms, after slaughterhouse and during processing was recommended, since broiler chickens are the main source of human *Campylobacter* infection.

Low microbial counts of *Campylobacter* were found in broiler chicken meats; only few samples showed higher microbial counts. However, the samples of broiler chicken meat with skin were the most contaminated. The study showed also that refrigeration could lethally injure and/or effectively reduce the microbial counts. The reduction on *Campylobacter* counts was greater in broiler chicken meat samples refrigerated storage with modified atmosphere packaging (MAP) than broiler chicken meat samples refrigerated storage with plastic film packaging. Furthermore, the presence of potentially virulent *C. jejuni* strains was found in broiler chicken meat at end shelf life after refrigerated storage, representing a potential risk to humans.

The results revealed a high occurrence of multidrug resistance among *Campylobacter* strains isolated both from broiler chickens in farms, at slaughterhouse and during processing. Particularly, high resistances were found to fluoroquinolones and macrolides, drugs of choice in the treatment of human campylobacteriosis.

High genetic diversity was observed among *Campylobacter* strains with the two techniques used in this study, s-AFLP and PFGE. Interestingly, several *Campylobacter* genotypes, found in this study, were previously identified and cited in the national database of Istituto Zooprofilattico of Abruzzo and Molise (IZS Teramo). The existence of specific genotype among *Campylobacter* strains isolated in different periods and from different Italian regions revealed stability of several genotypes to environmental pressures.

Application of strict biosecurity measures in broiler chicken farms and GMP/HACCP practices during slaughterhouse and during processing are recommended because may reduce *Campylobacter* colonization of broilers with consequent decrease *Campylobacter* numbers in the intestines, contamination of carcasses and retail poultry meats. Moreover the use of fly screens and of high quality water on farms, restriction of slaughter age of indoor farms to a max 28 days, or discontinued thinning, may reduce *Campylobacter* contamination in slaughterhouse and in retail broiler chicken meats. After slaughter, an estimated risk reduction of 100% can be achieved by irradiation or heat treatment, such as cooking, of broiler meat on an industrial scale. Moreover, a risk reduction of 87-98% can be reached by freezing carcasses for 2-3 weeks while a risk reduction of 37-96% can be obtained by short time freezing carcasses (2-3 days), hot water or chemical carcasses with lactic acid, acidified sodium chlorite or trisodium phosphate (Romero-Barrios *et al*., 2013).

Finally, a better handling raw broiler chicken in the kitchen, adequate hand hygiene, sanitization of countertops, cutting boards, cooking utensils before and after preparing food, and properly cooked poultry meat can reduce risk of illness caused by *Campylobacter*.

A future critical limit of 1000 *Campylobacter* bacteria/gram in neck skin would reduce the number of human disease cases by two-thirds. The costs to the poultry industry to meet this criterion (estimated at 2 million euro per year) are considerably lower than the averted costs of illness (approximately 9 million euro per year) (Swart *et al*., 2013).

CHAPTER 5. References

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