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ASSESSMENT OF ANTIBIOTIC AND BIOCIDE RESISTANCE IN *CAMPYLOBACTER JEJUNI* AND *CAMPYLOBACTER COLI* ISOLATES FROM HUMAN AND BROILER ORIGIN

Ana Filipa Moreira Martins

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Dissertação orientada pela Professora Doutora Maria João Fraqueza e pelo Professor Doutor Francisco Dionísio

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Abstract

In 2008 the European Food Safety Authority, EFSA, reported that *Campylobacter was* one of the most frequently observed bacterial gastrointestinal pathogen in humans. Poultry meat is an extremely important foodborne source of *Campylobacter* spp. Although self-limiting, campylobacteriosis occasionally requires treatment and macrolides and fluoroquinolones are the drugs of choice.

For the past few decades resistant *C. jejuni* and *C. coli* strains have emerged at an alarming rate due, at least partly, to the large-scale use of these agents in food production animals and passed on to humans through poultry meat products.

The purpose of this work was the phenotypic and genotypic characterization of isolates from two different origins: poultry and human, in order to perceive the general panorama regarding antimicrobial resistance for *C. jejuni* and *C. coli* strains in Portugal.

Disc diffusion tests results provided comprehensive information about antimicrobial resistance for the tested products and made selection of multiple drug resistant strains possible. Antibiotic and disinfectant minimum inhibitory concentrations were determined and the effect an efflux pump inhibitor in restoring ciprofloxacin susceptibility was analyzed. High resistance frequency to fluoroquinolones was observed reaching, for ciprofloxacin, levels as high as 92% (retail isolates), 30% to 40% of studied isolates were erythromycin resistant and resistance patterns for tetracycline ranged from 37% to 91%. Human isolates showed high level percentage of resistant isolates to both therapeutic alternatives. Multiple drug resistance was one of the major issues to be considered in the present work, which occurred in 86.3% to 93.2% of tested strains. Erythromycin and ciprofloxacin high level resistance (HLR) strains were among the selected isolates, and the respective HLR associated point mutations, were detected. The effect of the efflux pump inhibitor (PAβN) on ciprofloxacin resistance revealed that susceptibility to this antibiotic could be restored for mostly all, except for HLR ciprofloxacin strains.

Keywords: *Campylobacter,* fluoroquinolones, erythromycin, multiple-resistance, efflux pump inhibitors.

Resumo

Campylobacter spp. é reconhecido como um dos principais agentes responsáveis por gastroentrites de etiologia bacteriana em humanos. Entre as espécies deste género patogenicamente mais relevantes incluem-se *Campylobacter jejuni* e *Campylobacter coli* sendo que a primeira é responsável por cerca de 80 - 90% dos casos de campilobacteriose registados, enquanto os restantes casos são, na sua maioria, da responsabilidade de *C. coli* (Fitzgerald *et al., 2008*).

Tipicamente os casos de campilobacteriose são auto-limitantes e não requerem terapêutica antimicrobiana. Esta é apenas efectivamente necessária num relativamente reduzido número de situações: Caso a infecção ocorra em indivíduos imunocomprometidos, na eventualidade de se verificarem complicações secundárias extra intestinais ou em caso de persistência dos sintomas gastrointestinais (Nachamkin *et al.*, 1998; Skirrow *et al.*, 2000).

Os macrólidos e fluoroquinolonas surgem como os principais agentes terapêuticos indicados na resolução das situações anteriormente descritas. A resistência a estes agentes terapêuticos tem, no entanto, crescido a um ritmo alarmante nas últimas décadas verificando-se inclusivamente resistências simultâneas a diferentes grupos de antibióticos por parte de estirpes isoladas numa das principais fontes de infecção como sucede com a carne de aves (Vliet & Ketley, 2001; Gibreel *et al.*, 2006).

Os macrólidos são antibióticos bacterioestáticos que actuam por inibição da síntese proteica ligando-se à subunidade 23S rARN da subunidade ribossomal 50S, onde, perante a ocorrência de mutações pontuais, se verifica a alteração do alvo deste agente terapêutico inibindo assim a sua actividade. Estas mutações ocorrem ao nível do domínio V do gene 23S rARN, nas posições 2075 e 2074 ocorrendo, de forma geral, respectivamente uma transição (A2075G) e uma transversão (A2074C). Tipicamente esta primeira mutação pontual é predominante e será por isso um dos alvos neste estudo (Engberg *et al.*, 2001; Gibreel & Taylor, 2006).

As fluoroquinolonas são agentes antimicrobianos de largo espectro que actuam por inibição das topoisomerases. No caso de *Campylobacter* spp. é particularmente importante o seu efeito na topoisomerase II, ADN girase responsável pela manutenção do *supercoil* negativo da dupla hélice da molécula de ADN durante a replicação e transcrição. A ocorrência de uma mutação pontual ao nível da região determinante de resistência a quinolonas (QRDR, do ing. *Quinolone Resistance Determining Region*) afectando o aminoácido 86Thr (*Thr-Ile*), é o principal mecanismo de desenvolvimento de resistência a fluoroquinolonas. Não existem actualmente evidências da existência da topoisomerase IV em *Campylobacter* (Engberg *et al.*, 2001; Fàbrega *et al.*, 2008).

Para além da ocorrência de mutações pontuais observa-se o desenvolvimento de resistência, tanto a fluoroquinolonas como a macrólidos devido à actividade de mecanismos de efluxo. A bomba de efluxo CmeABC pertencente à família de transportadores RND (do ing. *Resistance Nodulation Division*), inicialmente descrita por Pumbwe e Piddock (2002) e Lin *et al.* (2002) encontra-se actualmente associada a mecanismos de resistência intrínseca e a baixos nível de resistência, LLR (do ing. *Low Level Resistance*) a estes agentes terapêuticos. A actividade da bomba de efluxo é inibida na presença do inibidor de bombas de efluxo, EPI (do ing., *efflux pump inhibitor*) (Webber & Piddock, 2003; Akiba *et al.*, 2006; Piddock, 2006).

O aumento de estirpes que apresentam resistência múltipla é actualmente uma relevante fonte de preocupação sob o ponto de vista clínico, sendo necessário determinar se a resistência apresentada por estirpes de origem animal se estende a outros antimicrobianos, nomeadamente desinfectantes uma vez que estes são utilizados frequentemente nos programas de higienização de matadouros, nas salas de desmancha de onde provêem as carcaças e peças de frango para consumo humano.

O objectivo deste trabalho prendia-se com a caracterização fenotípica e genotípica de isolados de *Campylobacter* de diferentes origens, amostras de produtos para consumo humano e amostras com origem em pacientes com campilobacteriose, de forma a captar o estado actual deste importante agente em relação a resistências a antimicrobianos tanto na carne para consumo humano como no próprio homem, em Portugal. Pretendia-se igualmente avaliar a resposta dos isolados a dois desinfectantes diferentes, utilizados rotineiramente na limpeza e desinfecção dos matadouros e salas de desmancha. Por fim era também pretendido apreciar o efeito de um inibidor de bomba de efluxo, PAβN (fenilalanina arginina β-naftilamida) no restabelecimento da susceptibilidade, neste caso especifico, à ciprofloxacina.

Tendo por base os objectivos descritos foram seguidas as metodologias que se consideraram mais adequadas. A identificação dos isolados foi inicialmente feita pelo teste do hipurato e posteriormente corroborada por PCR *multiplex*, de acordo com Dennis *et al*. (1999) e Samorsornsuk *et al*. (2007). Testes de susceptibilidade a antibióticos por difusão de disco foram realizados, de modo a proceder à elaboração dos perfis de resistência dos isolados obtidos, provenientes de amostras de frangos (pele do peito, intestino e peito de frangos de produção extensiva *indoor*, intensiva e biológica e de carcaças de frangos ‘pronto a cozinhar’ adquiridas em grandes superfícies comerciais) para 11 antibióticos, eritromicina, tetraciclina, gentamicina, amoxacilina e ácido clavulânico, ácido nalidixíco, ciprofloxacina, norfloxacina, ofloxacina, trimetropim-sulfametoxazole, cloranfenicol e ampicilina. A selecção de estirpes com resistência múltipla era então possivel. Ás estirpes seleccionadas for aplicada a técnica de concentração mínima inibitória, CMI, por diluição em agar, para eritromicina, ciprofloxacina, tetraciclina e gentamicina, assim como para os dois desinfectantes: hipoclorito de sódio e cloreto de benzalcónio. A gama de concentrações para os antibióticos está de acordo com o normativo *CA-SFM* 2010. A gama de concentrações a que os desinfectantes foram testados variou entre 0,5 a 32 µg/mL, de acordo com Peyrat *et al.* (2008).

A ocorrência de mutações pontuais, nomeadamente à mutação A2075G que surge ao nível do domínio V do gene responsável pela subunidade 23S rRNA, tipicamente perdominante em comparação à ocorrência da mutação A2074C na mesma região foi testada recorredo-se á técnica de RFLP-PCR (*Restriction Fragment Length Polymorphism*) de acordo com Kurinčič *et al.*, 2007. As mutações pontuais ao nível da região QRDR do gene responsável pela DNA girase, *gyr*A foram avaliadas pela técnica de MAMA-PCR (*Mismatch Amplification Mutation Assay*) de acordo com o trabalho de Payot *et al.* (2004).

O efeito da bomba de efluxo foi avaliado na presença de diferentes concentrações de ciprofloxacina e de diferentes concentrações de inibidor, PAβN (Phe-Arg-β-naftilamida) realizado de acordo com o estudo descrito por Payot *et al.* (2004).

Do numero total inicial de isolados, 75% tinham origem alimentar enquanto que os restantes tinham origem humana. Do número inicial de isolados, 258, 43% foram identificados como *C. jejuni* enquanto os restantes 57% foram identificados como *C. coli*.

Testes de difusão de disco providenciaram a informação necessária, relativamente à resistência a antibióticos, para os produtos testados. Foi possível constatar uma particular elevada frequência de resistência às fluoroquinolonas, 58% a 92%. Para o macrólido eritromicina, 30% a 40% dos isolados estudados eram resistentes. Foram também identificados níveis de resistência para a gentamicina e tetraciclina que variavam respectivamente entre: 6% a 45% e de 37% a 91%.

Isolados com origem em amostras de frangos de produção extensiva apresentaram, comparativamente aos isolados com origem em amostras de frangos de produção biológica, níveis mais baixos de resistência a ciprofloxacina enquanto os isolados de amostras de produtos prontos a cozinhar apresentavam os mais altos níveis de resistência a este antibiótico. O mesmo padrão de resistência foi observado para a tetraciclina onde se evidenciaram níveis de resistencia mais reduzidos para os isolados com origem em frangos de produção extensiva e os mais altos para isolados de amostras de produtos prontos a cozinhar. No caso especifico da eritromicina foi possível constatar, que os isolados de produtos prontos a cozinhar apresentavam-se mais susceptíveis (40% de isolados resistentes) que os isolados de frangos de produção extensiva (75% de isolados resistentes).

Os isolados de amostras de fezes humanas provenientes de indivíduos com campilobacteriose apresentavam níveis de resistencia particularmente elevados para as alternativas terapêuticas tipicamente utilizadas: 32% e 37% dos isolados eram resistentes respectivamente a eritromicina e à tetraciclina e mais de 70% eram resistentes à ciprofloxacina. Apenas 19% dos isolados apresentavam resistência a gentamicina, no entanto, devido à elevada toxicidade deste agente a sua aplicação é altamente limitada mas persiste enquanto alternativa terapêutica.

A questão da resistência múltipla era um ponto fulcral no desenvolvimento desta dissertação. Foi possível observar que para todos os isolados testados a maioria apresentava resistência para 4 ou mais das 7 classes de antibióticos testadas, tipicamente para quinolonas, macrólidos, tetraciclina e β-lactâmicos. Constatou-se que entre 86% e 93% das estirpes testadas apresentavam resistencia múltipla. Estirpes de origem humana e de origem em produtos prontos a cozinhar não apresentaram nenhuma estirpe totalmente susceptível a todas as classes de antibióticos testadas.

Todas as estirpes avaliadas como HLR para a ciprofloxacina e eritromicina eram portadoras das mutações pontuais, respectivamente *gyr*A (Thr86) e A2075G. As concentrações mínimas inibitórias mais altas foram obtidas para estirpes de origem em frangos de produção biológica. Para a eritromicina foi de 128 mg/L e de 512 mg/L para a ciprofloxacina. Foi também possível constatar algum paralelismo entre estirpes classificadas como HLR para a ciprofloxacina e os padrões de resistencia obtidos para os desinfectantes em estudo. As mais elevadas concentrações mínimas inibitórias foram de 64 mg/L para o cloreto de benzalcónio e de 512 mg/L para o hipoclorito de sódio. É relevante mencionar que as estirpes com origem em produtos prontos a cozinhar estudadas, apresentavam-se totalmente resistentes à actividade do hipoclorito de sódio.

Relativamente ao funcionamento da bomba de efluxo e à actividade do respectivo inibidor foi possível constatar que tipicamente a presença do inibidor devolvia inteiramente, até a concentração máxima de 40 mg/L, a susceptibilidade à ciprofloxacina. Para as estirpes classificadas como HLR, no entanto, a resistência a ciprofloxacina persistiu mesmo perante a concentração de inibidor mais alta testada: 120 mg/L.

È actualmente inquestionável a necessidade crescente de melhor compreender o processo subjacente ao desenvolvimento de resistências a antimicrobianos. O uso inadequado destes compostos levou ao aparecimento disseminado de estirpes resistentes associado a padrões de resistencia múltipla indicando a rápida perda de eficácia dos agentes actualmente disponíveis no tratamento de infecções em humanos. A necessária introdução de mecanismos de regulação e controlo deverá ser feita paralelamente à combinação de informações epidemiológicas disponíveis num contexto mundial em que Portugal não será a excepção.

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1. Introduction

1.1. General considerations

Thermotolerant *Campylobacter* spp., especially *Campylobacter jejuni* and *Campylobacter coli*, are considered to be the most frequent causative agents of human bacterial enteritis worldwide (Možina *et al.*, 2011), even though, according to the European Food Safety Authority (EFSA) the reported cases of human campylobacteriosis represent only a small fraction of the actual number that occur (Butzler *et al.*, 2004; EFSA Journal, 2010). *Campylobacter* is also recognized as a major contributing factor to Guillain-Barré syndrome, the most common cause of acute paralysis in both children and adults (Mena *et al.*, 2008).

Poultry meat is acknowledged as one of the most relevant food-borne sources of *Campylobacter* spp. human infection. The occurrence of this bacterium along the food processing chain, from flocks to processed poultry products sold to the consumers, is now the spotlight for applying control measures in order to restrict, as much as possible, the contact between human and *Campylobacter* spp. (Hermans *et al.*, 2011).

Typically the clinical syndrome associated with campylobacter infection resolves itself in a timely manner (Moore *et al.*, 2006). Still, on occasion, drug therapy is required. Macrolides, namely erythromycin and fluoroquinolones, such as ciprofloxacin are currently the main prescribed antimicrobial agents. The ever growing resistance patterns reported for such compounds as well as for cleaning and disinfection compounds used daily along the food processing chain is an alarming public health issue (Skirrow *et al.*, 2000; Moore *et al.*, 2006; Možina *et al.*, 2011).

Vicente *et al.* (2008) searched the PubMed database for the keywords *Campylobacter* and Portugal retrieving a mere seven publications, of which only one, published in 1992, reported results collected at the national level. Since then, several more articles have already been published concerning human *Campylobacter* infections in Portugal.

The purpose of this work was the phenotypic and genotypic characterization of isolates from different origins: extensive indoor, organic and intensive production systems, human stool samples from patients with campylobacteriosis and retail processed poultry products, in order to perceive the general panorama regarding antimicrobial resistance patterns for both poultry and human strains in Portugal.

1.2. The genus *Campylobacter*

In 1886, Theodor Escherich observed microorganisms in stool samples from children with diarrhea that morphologically resembled campylobacters. Later McFaydean and Stockman (1913) identified *Campylobacter*-like bacteria from fetal tissues in aborted sheep. Still, it was not until the 1970's, with the development of an appropriate selective growth media and the isolation of campylobacters from stool samples of patients with diarrhea, that

Campylobacter spp. became, at last, widely recognized as a human pathogen (Skirrow *et al.*, 1977; Altekruse *et al.*, 1999; Hänninen *et al.*, 2010). *Campylobacter* is now acknowledged as one of the most important causes of bacterial gastrointestinal infection in humans (Peterson *et al.*, 1994).

Campylobacter (from the Greek *campylo*, "curved" and *bacter*, "rod") belongs to the epsilon class of Proteobacteria, order Campylobacteriales. This order includes two other genera: *Helicobacter* and *Wolinella*. Like *Campylobacter*, members of this genus have relatively small genomes (1.6 to 2.0 mega bases) and can establish long-term associations with their hosts, sometimes with pathogenic consequences. The genus *Helicobacter* includes the specie *H. pylori* which, although able to be asymptomatically carried for decades, can cause gastric ulcers in humans and is therefore considered a pathogen. The genus *Wolinella* includes a single species, *W. succinogenes*, which colonizes cattle as a commensal organism (Young *et al.*, 2007).

Thirty two Campylobacter species have currently been described (Euzéby LBSN, http://www.bacterio.cict.fr/, accessed September 2011). *Campylobacter* spp. have been isolated from a large variety of different animals, and each *Campylobacter* species seems to favor certain animal species. Typically, members of the Campylobactereacea family are gram-negative, slender and spirally curved rods, small in size (0.2 – 0.5 µm) with a characteristic corckscrew-like darting motility by means of a single polar flagellum located at one or at both ends of the bacteria. They generally require low oxygen concentrations (3 to 15%) and carbon dioxide concentrations of 3 to 5%, hence being microaerophilic. *Campylobacter* spp. are fastidious organisms requiring complex growth media and are unable to ferment or oxidize carbohydrates, with the exception of L-fucose (Peterson *et al.*, 1994, Vliet & Ketley, 2001; Debruyne *et al.*, 2008; Stahl *et al.*, 2010).

The most important *Campylobacter* species are *C. jejuni*, accounting for over 90% of infections, and *C. coli* accounting for most of the remaining infections (Gibreel *et al.*, 2006). Growth temperature ranges from 34 to 44°C, with an optimal temperature of 42°C. This probably reflects the species adaptation to the intestines of warm-blooded animals, namely birds, which are the most frequent natural reservoirs. Natural reservoirs include both wild animals, namely migratory birds or rodents, and domestic animals such as poultry, pigs, ruminants, cats and dogs. These bacteria can also be found in natural water sources as well as in foodstuffs typically of animal origin, implicating poultry meat and raw milk as major sources of human infection (Vliet and Ketly, 2001). Cross contamination to vegetables has been described (Kumar *et al.*, 2000). Seldomly, humans can also transmit the disease to other humans by fecal-oral route (Altekruse *et al.*, 1999; Young *et al.*, 2007). Eating or handling undercooked chicken meat is considered one of the most important risk factors for human infection with reports stating that up to 90-100% of retail chicken meat world-wide is contaminated with *Campylobacter* spp. (Butzler *et al.*, 2004; Olson *et al.*, 2008; Možina *et al.*, 2011).

1.3. Epidemiological studies, *Campylobacter jejuni* and *Campylobacter coli*.

Epidemiological studies of *Campylobacter* infections indicate that there are two disease manifestations which are dependent on socio-economic status. In industrialized countries the incidence of *C.jejuni/C. coli* peaks during infancy and again in young adults aging from 15 – 44 years contributing primarily to morbidity (Butzler *et al.*, 2004). In the developing countries, children under the age of 5 are predominately affected but, severe manifestations of the infection are uncommon and isolation of *C. jejuni*, both from children with diarrhea as from those who are healthy, is extremely frequent (Blaser *et al.*, 1985; Young *et al.*, 2007).

The European Food Safety Authority and the European Centre for Disease Prevention and Control has analyzed the information on the occurrence of zoonoses and food-borne outbreaks in 2009, submitted by 27 European Union Member States (EU-MSs). Campylobacteriosis was the most commonly reported zoonosis, since 2005, with 198252 human cases. Reported campylobacteriosis case fatality rate was 0.02%, which is still lower than for salmonellosis. It is worth mentioning the absence of results concerning campylobacteriosis cases in humans for both Portugal and Greece (**Table 1**).

Seasonality has also been associated to the occurrence of campylobacteriosis. The highest numbers and notification rates of *Campylobacter* cases in humans were reported during the summer months, from June to August, and start gradually decreasing from September to December (EFSA Journal, 2011).

Antimicrobial resistance patterns have been previously described. An inevitable side effect of the use of antibiotics is the emergence and dissemination of resistant bacteria and resistance genes. Resistant bacteria from animals can infect the human population not only by direct contact but also via food products of animal origin (Fàbrega *et al.*, 2008). **Table 2** resumes the general panorama regarding ciprofloxacin and erythromycin resistance regarding several european countries.

Table 1 - Reported campylobacteriosis cases in humans 2005-2009 and notification rates for 2009. EFSA-EU summary report on trends and sources of zoonoses and zoonotic agents and food-borne outbreaks 2009, Published 22nd March 2011.

Country	Report Type[1]	2009			2008	2007	2006	2005
		Cases	Confirmed Cases	Confirmed cases/ 100,000	Confirmed cases			
Austria[5]	C	1,516	1,516	18.14	4,280	5,821	5,020	5,065
Belgium	C	5,697	5,697	53.41	5,111	5,906	5,771	6,879
Bulgaria	A	26	26	0.34	19	38	0	-
Cyprus	C	37	37	4.64	23	17	2	-
Czech Republic	C	20,370	20,259	193.54	20,067	24,137	22,571	30,268
Denmark	C	3,353	3,353	60.84	3,470	3,868	3,239	3,677
Estonia	C	170	170	12.68	154	114	124	124
Finland	C	4,050	4,050	76.04	4,453	4,107	3,439	4,002
France	C	3,956	3,956	6.15	3,424	3,058	2,675	2,049
Germany	C	62,331	62,331	76.01	64,731	66,107	52,035	62,114
Greece	-[4]	-	-	-	-	-	-	-
Hungary	C	6,583	6,579	65.59	5,516	5,809	6,807	8,288
Ireland	C	1,819	1,810	40.67	1,752	1,885	1,810	1,801
Italy	C	531	531	0.88	265	676	–	–
Latvia	U	0	0	0	0	0	0	0
Lithuania	C	812	812	24.24	762	564	624	694
Luxembourg	C	551	551	111.65	439	345	285	194
Malta	C	132	132	31.91	77	91	54	91
Netherlands[2]	C	3,782	3,739	43.62	3,341	3,289	3,186	3,761
Poland	C	357	357	0.94	257	192	156	47
Portugal	-[4]	-	-	-	-	-	-	-
Romania	C	328	254	1.18	2	-	-	-
Slovakia	C	3,902	3,813	70.45	3,064	3,380	2,718	2,204
Slovenia	C	952	952	46.84	898	1,127	944	-
Spain[3]	C	5,106	5,106	44.57	5,160	5,055	5,889	5,513
Sweden	C	7,178	7,178	77.55	7,692	7,106	6,078	5,969
United Kingdom	C	65,043	65,043	106.32	55,609	57,815	52,134	52,686
EU Total		**198,582**	**198,252**	**45.57**	**190,566**	**200,507**	**175,561**	**195,426**
Iceland	C	74	74	23.17	98	93	117	128
Liechtenstein	-	-	-	-	2	0	10	-
Norway	C	2,848	2,848	59.34	2,875	2,836	2,588	2,631
Switzerland	C	8,154	8,154	105.90	7,817	6,038	5,429	5,259

1. A: aggregated data report; C: case-based report; –: No report; U: unspecified.
2. Sentinel system; notification rates calculated on estimated coverage 52 %.
3. Sistema de informacion microbiologica (SIM); notification rates calculated on estimated coverage 25 %.
4. No surveillance system exists.
5. New electronic reporting system in place since 2009.

Table 2 - General panorama regarding *Campylobacter jejuni* and/or *Campylobacter coli* resistance to ciprofloxacin and erythromycin for some relevant european countries. CIP, ciprofloxacin; ERY, erythromycin.

Country	Strain origin	Tested strains	*Campylobacter spp.*		*C.jejuni*		*C.coli*		Ref.
			CIP	ERY	CIP	ERY	CIP	ERY	
France	Human / broiler	1135	-	-	25.3%/ 9.4%	1.4%/ 0%	42%/ 32.4%	12.5%/ 17.6%	Gallay *et al.*, 2007
Switzerland	Poultry (retail raw meat)	91	28.7%	1.1%	-	-	-	-	Lederberger *et al.*, 2003
Slovenia	Poultry (retail raw meat)	55	58.2%	14.5%	-	-	-	-	Kurinčič *et al.*, 2005
Finland	Human	226	59.3%	-	-	-	-	-	Lehtopolku *et al.*, 2005
Portugal	Poultry retail meat	99	90%	90%	-	-	-	-	Mena *et al.*, 2008
Portugal	Human	123	80.5%	-	-	-	-	-	Vicente *et al.*, 2008
Denmark	Human / broiler	93	-	-	24%/ 0%	0%/ 0%	-	-	Danmap *et al.*, 2009
Italy	Human / broiler	309	-	-	38.2%/ 42.2%	1.4%/ 3.1%	55.2%/ 75%	24.1%/ 45%	Pezzotti *et al.*, 2003
Spain	Human / broiler	754	-	-	75%/ 98.7%	3.2%/ 20.3%	70.7%/ 100%	34.5%/ 90%	Saenz et at., 2000
Germany	Human / broiler	509	-	-	46.2%/ 42%	0%/ 0.8%	41.2%/ 70.6%	29.4%/ 5.9%	Luber *et al.*, 2003
Sweden	Human / broiler	208	6% / 2%	0% / 0%	-	-	-	-	Rönner *et al.*, 2004
Ireland	Human / Chicken	377	17.4%/ 4.5%	4.2%/ 11.4%	-	-	-	-	Moore *et al.*, 2001

1.4. Pathology and disease

Many questions are still open concerning campylobacters pathogenic mechanisms but many have already been understood. To establish an infection, as other pathogenic bacteria, *C. jejuni*, in association with food or water must bypass both mechanical and immunological barriers of the gastrointestinal tract in order to enter the host intestine, via the stomach acid barrier, and colonize the distal ileum and colon. Following colonization of the mucus and adhesion to intestinal cell surfaces, campylobacters perturb the normal absorptive capacity of the intestine by damaging epithelial cell function either directly, by cell invasion or toxin(s) production, or indirectly, by initiating an inflammatory response. As these possible mechanisms are not mutually exclusive, any combination may have a role depending on the host status and attributes of the infecting strain (Ketley, 1997).

Campylobacter enteritis is an acute diarrheal disease with clinical manifestations like those of other bacterial gut infections that distress the intestinal tract, such as salmonellosis or shigellosis. Except for the fact that in humans, *C. jejuni* is responsible for the great majority of enteric *Campylobacter* infections (80–90%), leaving *C. coli* responsible for most of the remaining 10 to 20% which can also be ascribed, in a lower proportion, to *C. lari* and *C. upsaliensis.*There does not seem to be any greater differences between the clinical outcome of infections caused by either *C.jejuni* or *C. coli* (Fitzgerald *et al., 2008*).

Generally, the infective *Campylobacter* dose is low. Infection has been induced with doses as low as 500 bacteria and this number may even be lower when milk or water-borne contamination occurs due to the buffering and/or "wash-through" effect throughout the stomach contents. The incubation period ranges from 18h to 8 days being 3.2 days the average incubation period (Skirrow *et al.*, 2000).

The clinical outcome of *Campylobacter* contamination is determined by the virulence of the infecting strain, the challenge dose and also on the patients susceptibility (health status, age). The onset of the disease is often abrupt starting with severe abdominal pain followed by profuse, watery, bile stained and sometimes prostrating diarrhea. Blood in stool may occur as infection progresses into the colon and rectum (**Table 3**). Severe symptoms do regress after 3 to 4 days into the illness but patients continue to excrete campylobacters

Table 3 - Clinical features of *Campylobacter* enteritis derived from surveys of community outbreaks in which or more people were affected and analyzed. (Skirrow *et al.*, 2000).

Symptom	**Frequency (%)**	
	Mean/median	**Range**
Fever (25)	50/52	6-75
Diarrhea (26)	84/85	52-100
Headache (21)	41/47	6-69
Abdominal pain (26)	79/80	56-99
Myalgia (5)	42/37	28-59
Vomiting (20)	15/11	1-42
Blood in feces (7)	17/13	0,5-32

in their faeces for several weeks after they have clinically recovered, unless the antibiotic treatment took place (Skirrow *et al.*, 2000).

Generally, the illness is self-limiting completing resolving itself in a timely manner. Although less frequently, *Campylobacter* infection may produce other, more severe outcomes, resulting in severe intestinal compromise or even extraintestinal complications such as bacteremia, septic arthritis, Reiter's syndrome or Guillian-Barré syndrome (GBS), amongst others. The latter is the most serious post-*Campylobacter* infection complication, occurring in 1 out of every 1000 campylobacteriosis affected individuals with symptoms beginning 1 to 3 weeks after the onset of *Campylobacter* enteritis. *Campylobacter* associated GBS cases results in a usually severe disorder of the peripheral nervous system characterized by ascending symmetrical weakness and eventually paralysis requiring intensive hospital treatment with a possibility of long-term disability or even a fatal outcome. Although not well defined, some investigators have reported that GBS following *Campylobacter* infection may be more severe and result in more irreversible neurologic damage than GBS following other putative infections (Nachamkin *et al.*, 1998). The autoimmune response underlying GBS owes itself to the occurrence of molecular mimicry between various *C. jejuni* LOS (lipooligosaccharide) structures and human neuronal gangliosides (Nachamkin *et al.*, 1998).

Still, having in general a very good prognosis, no specific treatment is required for the majority of patients with C*ampylobacter* enteritis other than supportive measures such as fluid and electrolyte replacement. Antibiotic therapy is only advised for patients acutely ill with enteritis, persistent fever or suffering from either prolonged or bloody diarrhea. HIV (human immunodeficiency virus) positive or other immunocompromised individuals should initiate antibiotic treatment as soon as possible for the outcome, when therapy is delayed, may not turn out to be as successful (Altekruse *et al.*, 1999; Skirrow *et al.*, 2000)

1.5. Antibiotic therapy: Selected antibiotics for campylobacteriosis management

The introduction of antibiotics was one of the most important medical interventions regarding the reduction of human morbidity and mortality. The discovery of penicillin in 1928 by Alexander Fleming was the beginning of modern antibiotic medicine. Mass production started in the 1940s, and today hundreds of antimicrobials are available on the market. An estimated half of the globally produced antimicrobials are used for food animals (World Health Organization, 2009).

Antibiotics can be classified based on the cellular component or system they affect in addition to whether they induce cell death (bactericidal effect), or merely inhibit cell growth (bacteriostatic effect). Most bactericidal antimicrobials act by inhibiting DNA, RNA, cell wall or protein synthesis (Kohanski *et al.*, 2010). They can be synthetic or semi-synthetic compounds or natural substances produced by other microorganisms.

The intensive use of antibiotics since the 1940s, has dramatically increased the frequency of resistance among human pathogens and threatens a loss of therapeutic options and an upsurge of a post-antibiotic era in which the medical advances to date are negated. Resistance dramatically reduces the possibility of treating infections effectively and increases the risk of complications and of a fatal conclusion. Every year in the European Union alone it is estimated that over 25000 people die of antibiotic-resistant bacterial infections, mostly acquired in hospitals (Andersson & Hughes, 2010; World Health Organization, 2011).

As previously mentioned, most cases of enteritis do not require treatment as they are of short duration, clinically mild and self limiting. However, antimicrobial therapy is imperative when long-lasting infections and/or systemic compromise after *Campylobacter* infections occur or when infection arises in immune-suppressed patients. Two major classes of antibiotics are taken under consideration regarding the treatment of *Campylobacter* induced infection. The macrolide Erythromycin is considered the drug of choice for treating Campylobacter gastroenteritis and ciprofloxacin and tetracycline are used as alternative drugs. Eventually aminoglycosides such as gentamicin can be used for serious systemic infections (Skirrow *et al.*, 2000; Alonso *et al*., 2005; Gibreel *et al.*, 2006)

1.5.1.Macrolides: Erythromycin

The term “macrolide” is used to describe drugs with a macrocyclic lactone ring of 12 or more elements. This class of compounds includes a variety of bioactive agents, including antibiotics. The 14-, 15-, and 16-membered macrolides are a widely used family of antibiotics (Ladely *et al.*, 2009). They have excellent tissue penetration and antimicrobial activity, mainly against Gram-positive cocci and atypical pathogens. The macrolide antibiotics include natural members, prodrugs and semi-synthetic derivatives. Erythromycin A, a 14-membered macrolide, was isolated from cultures of *Streptomyces erythreus* and was the first macrolide introduced into clinical practice, being approved by the FDA in 1959 (Piretti *et al.*, 1992). Later on, in 1991, azythromycin was FDA approved. This semi-synthetic macrolide is similar in structure to erythromycin but has a better farmacokinetic proprieties than the latter, considering oral bioavailability, tissue penetration and persistence, and long elimination half-lives, which allow for once-daily or twice-daily dosing however, azithromycin does not exhibit increased potency in comparison with erythromycin (Piscitelli *et al.*, 1992; Gibreel *et al.*, 2007).

Erythromycin is a bacteriostatic agent that acts as a protein synthesis inhibitor (Taylor & Tracz, 1991). Erythromycin attaches itself, through hydrogen bonds between the macrolide desosamine sugar group and the polar groups on specific nucleotides: 2058 and 2059 (*Escherichia coli* numbering) causing the dissociation of the peptidyl-tRNA in the 50S tunnel where elongation takes place (**Figure 1**) (Engberg *et al.*, 2001, Ladely *et al.*, 2009). The occurrence of point mutations within the peptidyl-transferase region, in domain V of the 23S rRNA target gene, which is the main anchoring point for macrolides, prevents erythromycin from

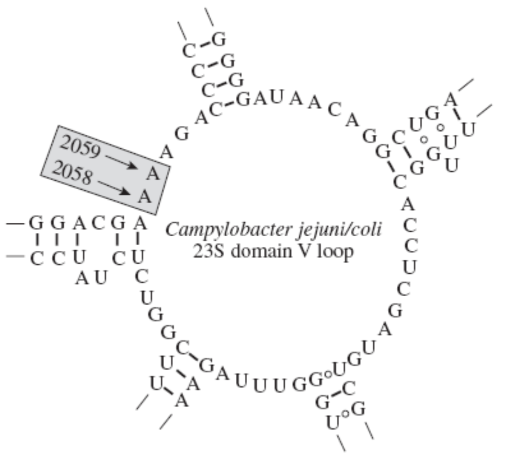

Figure 1 - Secondary structure of the peptidyl-transferase loop in domain V of C. jejuni/coli 23S rRNA. Positions 2058 and 2059 (based on *E. coli* numbering) refer to the location of the mutations associated with erythromycin resistance, corresponding to positions 2074 and 2075 for *C. jeuni*. From Gibreel & Taylor, 2006.

attaching. In the majority of macrolide-resistant *C. jejuni* isolates, the resistance-associated mutations were identified in the three chromosomal copies of the target gene, however no correlation was observed between the level of macrolide resistance in *Campylobacter* isolates and the number of mutated copies of the 23S rRNA gene (Gibreel & Taylor, 2006; Ladely *et al.*, 2009). Still, at least two mutated copies are necessary to confer macrolide resistance and both the wild-type and mutated alleles can coexist in a single macrolide-resistant mutant (Gibreel & Taylor, 2006; Hao *et al.*, 2009). So far, described point mutations occur at positions 2074 and 2075 of the 23S rRNA. The latter, an A→G transition, is the most frequently observed. This point mutation is usually associated with high-level erythromycin resistance phenotypic expression. Mutations occurring in position 2074, include the transition, A→G which is rare and somewhat unstable (Gibreel & Taylor, 2006). And the transversion, A→C, which is not only correlated with stable and high-level macrolide resistance, having even higher erythromycin minimum inhibitory concentrations than isolates with the A2075G transition, but also associated with a fitness cost (Hao *et al.*, 2009). A recent finding demonstrated that modifications in the ribosomal proteins L4 (V4E and V196A) and L22 (G86E) (insertions at position 86 or 98) also conferred macrolide resistance in Campylobacter (Hao *et al.*, 2009).

1.5.2.Fluoroquinolones: Ciprofloxacin

Unlike some of the first discovered antibiotics, the quinolone class of antimicrobial agents was not isolated from living organisms but, rather synthesized by chemists. Nalidixic acid, a by-product of the synthesis of the antimalarial compound chloroquine was accidentally discovered in 1962 by Leser and his collegues and introduced in the same decade to treat urinary tract infections. Nalidixic acid and other first generation quinolones are rarely used today due to their toxicity. Second (ciprofloxacin), third (levofloxacin) and fourth (gemifloxacin) generation quinolone antibiotics can be classified on the basis of their chemical structure and of qualitative differences between the killing mechanisms they use. (Andriole, 2005; Kohanski *et al.*, 2010).

Quinolones are classified as nucleic acid inhibitors due to their interference with the modulation of chromosomal supercoiling by meddling with the topoisomerase-catalysed strand breakage and rejoining reactions. DNA *gyrase* induces negative supercoils in the DNA while topoisomerase IV is important in unlinking activity, making both enzymes essential. Thus cessation of their activities results in cessation of bacterial growth and death (Kohanski *et al.*,2010).

Fluoroquinolone resistance in *Campylobacter* spp. appears to be due to the occurrence of mutations in the genes encoding subunits of DNA gyrase. The GyrA and GyrB subunits of DNA gyrase are respectively homologous with the ParC and ParE subunits of topoisomerase IV although *Campylobacter*, as well as other microorganisms such as *Helicobacter pylori* and *Mycobacterium tuberculosis*, do not have topoisomerase IV (Engberg *et al.*, 2001; Fàbrega *et al.*, 2008). Cloning and sequencing of *C. jejuni gyr*A gene demonstrated that mutations in *gyrA* at positions Thr-86, Asp-90, Ala-70 and Pro-104 were responsible for fluoroquinolone resistance. The first mentioned is associated with higher level resistance to nalidixic acid and ciprofloxacin than the alternative mutations (**Table 4**) (Engberg *et al.*, 2001).

Table 4 - Fluoroquinolone resistance mechanisms reported in *Campylobacter* species (Adapted from Engberg *et al.*, 2001).

Fluoroquinolones	⟶	***gyrA***	**Thr-86** (Higher MIC to fluoroquinolones)
			Asp-90, **Ala-70** and **Pro-104** (Lower MIC to fluoroquinolones)

1.5.3.Tetracyclines: Tetracycline

The tetracyclines, which were discovered in the 1940s, are a family of antibiotics that inhibit protein synthesis by preventing protein translation by inhibiting the aminoacyl-tRNA binding to the ribosome (Chopra & Roberts, 2001; Kohanski *et al.*, 2010). Tetracyclines are broad-spectrum agents with no major adverse side effects which has led to the extensive use in therapy of both human and animal infections (Chopra & Roberts, 2001). Tetracycline high-level resistance is primarly mediated by the plasmid encoded gene, *tet(O)*, which encodes for the ribossomal protection protein Tet(O). This protein is structurally related to the translation ribossome-binding proteins, and acts by displacing tetracycline from its primary binding site on the ribossome (Chopra & Roberts, 2001; Pratt & Korolik, 2005).

1.5.4.Aminoglycosides: Gentamicin

Aminoglycosides are highly potent, broad-spectrum antibiotics. In 1944 streptomycin was the first to be attained, followed by other relevant compounds such as kanamycin, gentamicin, and tobramycin which definitively established the usefulness of this class of antibiotics for the treatment of gram-negative bacillary infections (Mingeot-Leclercq *et al.*, 1999).

Aminoglycosides act primarily by impairing bacterial protein synthesis through binding to prokaryotic ribosomes, namely the 16S rRNA component of the 30S ribosome subunit (Mingeot-Leclercq *et al.*, 1999; Kohanski *et al.*, 2010). Passage of these highly polar molecules across the outer membrane of gram-negative bacteria typically involves the drug-induced disruption of Mg^{2+} bridges between adjacent lipopolysaccharides and the subsequent transport of aminoglycosides across the cytoplasmic membrane which is dependent of electron transport. Upon reaching the cytosol aminoglycosides perturb the elongation of the nascent chain by impairing the proofreading process controlling translational accuracy (misreading and/or premature termination). The aberrant proteins may be inserted into the cell membrane, leading to altered permeability and further stimulation of aminoglycoside transport (Mingeot-Leclercq *et al.*, 1999).

1.6. The efflux pump – efflux pump inhibitors

Efflux pumps are transport proteins involved in the extrusion of toxic substances from within cells into the external environment. These pumps may be specific for one substrate or may transport a relatively wide range of structurally dissimilar compounds, including antibiotics of different classes. In gram-negative bacteria three mechanisms of resistance have been described which include drug inactivation, active efflux and modification of the target sites by methylation or mutation. Until recently little was known about efflux systems in *Campylobacter* spp., the first approach on the subject was made by Charvalos *et al.* (1995) who pinpointed the existence and contribution to multidrug resistance of a promiscuous efflux system (Lin *et al.*, 2002).

In the prokaryotic kingdom there are five major families of efflux transporters: MF (major facilitator), MATE (multidrug and toxic efflux), RND (resistance-nodulation-division), SMR (small multidrug resistance) and ABC (ATP binding cassette) (**Figure 2**). All these systems utilize the proton motive force as an energy source apart from the ABC family, which utilizes ATP hydrolysis to drive the exportation of substrates (Webber & Piddock, 2003; Piddock, 2006).

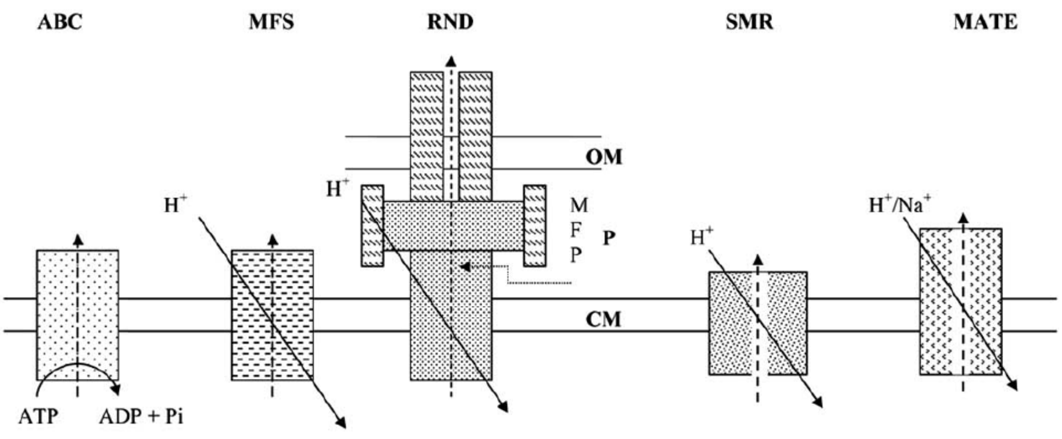

Figure 2 -. Diagrammatic representation of the five structural classes of bacterial efflux pumps. ABC: ATB binding cassettes, MFS: major facilitator superfamily, RND: resistance-nodulation-division, SMR: small multidrug resistance, MATE: multidrug and toxic compound extrusion, OM: outer membrane, P: periplasm, CM: cytoplasmic membrane, MFP: membrane fusion protein. (Adapted from Zechini & Versace, 2009).

One important family of drug transporters in *Campylobacter* spp. are the resistance nodulation cell division (RND) efflux systems. Genetically, many of the RND-type efflux systems or pumps are encoded by a three-gene operon located at the bacterial chromosome and the expression of these pumps is controlled by regulatory proteins. The efflux pump identified for *Campylobacter* spp. follows such expected conditions. CmeABC is a tripartite efflux system consisting of a periplasmic fusion protein (CmeA), an inner membrane drug transporter (CmeB) and an outer membrane protein (CmeC). CmeR is a transcriptional repressor that controls the expression of the mentioned pump and is encoded by a gene located immediately upstream of CmeABC. CmeR binds to the inverted repeat in the promoter region of CmeABC and inhibits the expression of the efflux operon (Akiba *et al.*, 2006).

CmeABC is essential for *Campylobacter* colonization *in vivo* by mediating resistance to bile salts normally present in the intestinal tract of animals. Besides bile salts, the pump allows the extrusion of a wide variety of compounds from dyes, heavy metals, detergents and antibiotics of different families thus contributing to multidrug resistance. In gram-negative bacteria, chromosomally encoded efflux pumps contribute to intrinsic resistance to a variety of antimicrobial agents, including macrolides, contributing to low level resistance. And although high levels of resistance may not occur as a result of MDR efflux pumps, the multidrug efflux pump works synergistically with other non-efflux resistance mechanisms such as target mutations to confer high levels of antimicrobial resistance in bacteria (Lin *et al.*, 2002; Webber & Piddock, 2003; Mamelli *et al.*, 2005; Akiba *et al.*, 2006). In addition to CmeABC, *Campylobacter* spp. possesses another RND-type efflux pump CmeDEF, in which CmeD, CmeE and CmeF are predicted to be an outer membrane channel protein, a periplasmic fusion protein and inner membrane transporter, respectively. CmeDEF is expressed in low levels and acts interactively with CmeABC in conferring resistance to antimicrobials and toxic compounds (Akiba *et al.*, 2006)

1.7. Antibiotic growth promoters and antibiotic resistance

The growth promoter effect of antibiotics was discovered in the 1940s, when it was observed that animals fed with dried mycelia of *Streptomyces aureofaciens*, containing chlortetracycline residues, had growth improvements (Castanon, 2007). Antibiotics are used in veterinary medicine for treatment and prevention of infections and as growth promoters in food animals.
The term "antibiotic growth promoter" or AGP is used to describe any medicine that destroys or inhibits bacteria and is administered at a low, sub- therapeutic dose in order to help growing animals digest their food more efficiently, without the lost of energy diverted for microbial fermentation and thus getting the maximum benefit from it allowing for a faster development into strong and healthy individuals. If the microbial population is better controlled, it is possible that the lost energy could be diverted to the animal growth (NOAH, 2001; Hughes & Heritage, 2002; Soulsby, 2007).

Early concerns about the impact of extensive utilization of AGPs could have on human health appeared in 1951 but it was not until 1969 that recommendations to ban the subtherapeutic use of human medicine antibiotics in animal feeds were discussed. The first nation to eliminate the use of antimicrobials for growth promotion was Sweden in 1986. In 1997 avoparicin, a glycopeptide growth promoter was banned due to reports on glycopeptide- resistant enterococci (GRE) isolated from food animals in England. Although no connection was made between the resistance in bacteria from food animals and infection in humans, the concern that its use created an animal reservoir of GRE and that this was a potential risk to public health remained. Two years later, other individual growth promoters were banned by the EU Commission because they belonged to classes of antimicrobials also used in humans (tylosin, spiramycin, bacitracin, and virginiamycin) or were considered unacceptable due to occupational toxicity risks (olaquindox and carbadox). Denmark has restricted the use of antimicrobials to therapeutic use, by prescription only, since January 2000.

European Regulation Concerning Additives in Animal Feeds Council published the 70/524 directive, in the Official Journal L270 of December 14, 1970, the basic principle of this regulation was that only those additives which are named in the Directive may be contained in foodstuffs. The member states, brought into force the laws, regulations, and administrative provisions necessary to comply with this Directive within 2 years following the notification. Directive 70/524 was later replaced by Regulation 1831/2003 of the European Parliament and of the Council on additives for use in animal nutrition. This Regulation stated that antibiotics, other than coccidiostats and histomonostats (antiprotozoan agents), might be marketed and used as feed additives only until December 31, 2005. Anticoccidial substances, such as antibiotics ionophores, also will be prohibited as feed additives before 2013. After this date, medical substances in animal feeds will be limited to therapeutic use by veterinary prescription (Castanon, 2007).

1.8. Disinfectants

Although highly fastidious and stress-sensitive organisms, *Campylobacter* is able to survive in the slaughterhouse environment overcoming cleaning procedures. During slaughter in slaughterhouses the contents of the birds GI tract may spill during evisceration contaminating the carcasses. Although little information is available about *Campylobacter* susceptibility to disinfectants, it is generally considered susceptible to the disinfectants used in the food industry. Yet, the concern that the increasing use of chemical disinfection, particularly of quaternary ammonium and chlorine compounds, might contribute to the emergence of disinfectant-resistant microorganisms and the simultaneous cross-selection for antibiotic resistant of the same strains has been mentioned representing a public health burden (Moore *et al.*, 2006; Peyrat *et al*., 2008).

Typically, cleaning and disinfection, C&D, procedures are relatively standardized and performed daily. They start in all cases with removal of the organic matter with high-pressure water, then application of detergent and disinfectant molecules to surfaces presumed free of organic matter.

Campylobacter is usually considered susceptible to the disinfectant molecules used in the food industry still this bacterium is still routinely detected in floor surface swabs of commercial transport cages after C&D (Peyrat *et al.*, 2007; Peyrat *et al.*, 2008). The frequency of strains with a low-level resistance to quaternary ammonium compounds (QAC) is relatively high for other foodborne pathogens such as *Listeria monocytogenes* (10%), *Staphylococcus aureus* (13%) or spoilage bacteria such as *Pseudomonas* spp. (30%) isolated from food and food processing industry. In general, works performed in other bacteria led to the conclusion that bacteria isolated after disinfection are more resistant although no link between resistance to disinfectants and antibiotics has been observed for *Campylobacter*. Yet, a genetic linkage has been found between resistance to QAC and antibiotics in food associated *Staphylococci*, and there is a growing concern about cross-resistance between antibiotics and disinfectants (Moore *et al.*, 2008). There is therefore a pressing need of developing more efficient C&D strategies and a better understanding of the mechanisms underlying the survival and resistance of *Campylobacter* in the environment limiting the public health burden the situation imposes (Peyrat *et al.*, 2007; Peyrat *et al.*, 2008).

1.9. Poultry – production systems

Poultry farming is the practice of raising poultry, such as chickens, turkeys, ducks and geese for the purpose of farming meat or eggs for human consumption. Broilers, chickens farmed for meat purposes, are generally held in large groups either in environmentally controlled housing or in open, naturally ventilated poultry houses. Broilers are usually kept free on deep litter with automated provision of feed and water. In most countries, commercial breeds selected for rapid growth, are used. Farmers around the world understand that in order to raise the birds with maximum efficiency, many conditions must be fulfilled – stress prevention, supply of good feed and water, and good sanitation. In providing these conditions, farmers ensure a basic level of animal welfare (Horne & Achterbosch, 2008).

Several farming techniques are currently used for poultry production: organic, extensive indoor and intensive production system. Organic production systems require a maximum of 10 birds per m^2 with mandatory access to access an outdoor range (4 m^2/bird), organic feeding is required. Organic chickens are slow growers; more traditional breeds are used and live typically for around 81 days till slaughter. Extensive indoor production systems imply that 12-14 birds are maintained per m^2, poultry are raised on pasture, enabling the poultry to move around and forage for their natural diet. Extensive indoor production allows broilers to live typically for 56- 81 days before slaughter. Meat chickens subjected to intensive production system are floor-raised on litter such as wood shavings or rice hulls, and kept indoors in climate-controlled housing. Poultry producers routinely use legally approved medications, in feed or drinking water, to treat disease or to prevent disease outbreaks, no outdoor access is required and animals will live less than six weeks before slaughter (Fuller, 2004).

1.10. Aims of the study

Given the general panorama regarding the occurrence of *Campylobacter*, namely *Campylobacter jejuni* and *Campylobacter coli*, along the food processing chain, from broiler flocks till processed retail products, and the human health consequences such a situation imposes the need to make notification mandatory. Not only due to its prevalence, but also due to the ever growing levels of resistance reported for *Campylobacter* in paralel with scarce results and notification rates that Portugal currently possesses for both foodstuffs and more importantly in humans. It was only relevant to adress the matter at hand by providing a new set of results regarding antibiotic and biocide resistance for *C.jejuni* and *C. coli* from human and broiler origin.

The purpose was this study was to performe susceptibility testing, using 7 different classes of antibiotics, for target isolates in order to understand the general panorama concerning resistant profiles showed by the tested strains as representative strains and then to select those that showed a multiple resistant profile. Selected strains were then re-tested for antibiotic and disinfectant resistant patterns and for the detection of point mutations to with resistance is highly attributed. The effect of an efflux pump inhibitor, to which restored susceptibility has been described was also studied.

2. Materials and Methods

2.1. *Campylobacter* isolates collection

The *Campylobacter* isolates (N = 258) used in the present study belonged to the collection of the *Laboratório de Tecnologia e Segurança dos Alimentos* at the *Faculdade de Medicina Veterinária da Universidade Técnica de Lisboa*. Isolates were attained from samples of different origins:

a. Caecum, carcass (neck skin) and breast meat were sampled at a poultry slaughterhouse, considering flocks' traceability for organic, extensive indoor and intensive production system in different days in 2008.
b. Retail samples of different processed poultry products, ready to cook and packaged under modified atmosphere/MAP (poultry deboned pieces, steaks, fresh sausages, burgers, balls, stroganoff, hot wings, marinated poultry meat and seasoned poultry meat) from different industrial units, were collected from October to December 2009,
c. Human campylobacter isolates, kindly provided by the *Instituto Nacional Saúde Dr. Ricardo Jorge*, were attained from patients with campylobacteriosis during 2008 and 2009. Strains from human origin were thus received isolated and purified in Columbia blood agar plates adequately prepared for transportation between institutions.

2.2. *Campylobacter* isolation and identification.

Campylobacter isolation was performed according to ISO10272-1:2005. Presumptive genus identification of the isolates was performed by: colony observation, observation of cell morphology, Gram staining and catalase and oxidase production. Hipurate hydrolysis allowed for the initial differentiation between *C. jejuni* and *C.* non-*jejuni* strains. *C. jejuni* and *C. coli* identification was performed after DNA extraction (Quiagen Kit DNA extraction (Qiagen, Valencia, CA, USA) or Pitcher *et al.* (1999)), by multiplex-PCR according to Dennis *et al.*, (1999) or for *cdtA*, *cdtB* and *cdtC* according to Samorsornsuk *et al.* (2007).

2.3. Reference strains

The reference strains *C. jejuni* K49/4 and ZIM 375/06, *C. coli* ZIM 140 were kindly provided by Prof. Sonja Možina from the University of Ljubljana, Lithuania. *C. jejuni* NCTC11168 was kindly provided by Prof. Naoaki Misawa from Japan. *Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212 belong to the *Faculdade de Medicina Veterinaria* collection and were used as disc susceptibility and minimum inhibitory concentration testing controls.

2.4. Storage and preservation of bacterial strains

Campylobacter isolates and reference strains were cryogenically preserved in Brain Hearth Infusion (Scharlau) with 30% glycerol (Scharlau) and were frozen at -80ºC (ThermoFisher Scientific, USA).

2.5. Bacterial growth conditions

Bacterial strains were grown on Columbia Blood agar (BioMerieux), supplemented with 5% horse blood and incubated at 42°C for 48h under microaerobic conditions (5% O_2, 10% CO_2 and 85% N_2) in gas tight containers.

2.6. Agar disc diffusion method

The agar disc diffusion method is a widely used, fast and a relatively low-cost, method for screening antimicrobial susceptibility that provides results classifying isolates as resistant, intermediate or susceptible (Wiegand *et al.*, 2008). For this test, an appropriate culture medium, Mueller-Hinton agar, was uniformly inoculated with the test organism. Filter paper discs, which impregnated with a specific concentration of a particular antibiotic, were placed on the medium. The organism grew on the agar plate while the antibiotic potentially inhibits the growth. Disc diffusion susceptibility testing was performed according to *Comité de l'antibiogramme de la Société Française de Microbiologie (CA-SFM)* and *European Committee on Antimicrobial Susceptibility Testing (EUCAST)* for eleven antibiotics of 7 different antibiotic classes (**Table 5**): Ampicilin (10µg); erythromicin (15 µg), tetracycline (30 µg); chloramphenicol (30 µg); gentamicin (10 µg); ciprofloxacin (5 µg), nalidixic acid (10 µg); norfloxacin (5 µg); ofloxacin (5 µg); amoxacilin + clavulanic acid (20 + 10 µg) and trimethropim + sulfametoxazole (25 µg). Strains were classified as resistant, intermediate or susceptible according to *CA-SFM*, 2011. For intensive production poultry origin isolates disc diffusion testing was not performed because the data had already been published by Borges (2009).

Table 5 - List of all antibiotics and respective concentrations used in agar disc diffusion susceptibility testing of all strains.

Antibiotic type (class)	Antibiotic agents	Disk concentration	Supplier
β-lactam	Ampicillin	*10µg*	Liofilchem
	Amoxacilin + clavulanic acid	*20 + 10µg*	Liofilchem
Macrolide	Erythromycin	*15µg*	Liofilchem
Tetracyclines	Tetracycline	*30µg*	Liofilchem
Phenicols	Chloramphenicol	*30µg*	Liofilchem
Aminoglycosides	Gentamicin	*10µg*	Liofilchem
Fluoroquinolone	Ciprofloxacin	*5µg*	Liofilchem
	Nalidixic acid	*10µg*	Liofilchem
	Norfloxacin	*5µg*	Liofilchem
	Ofloxacin	*5µg*	Liofilchem
Trimethoprim/ sulfamethoxazole	Trimethoprim/sulfamethoxazole	*25µg*	Oxoid

Quality control (QC) strains *Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212, for which quality control ranges have been established, were included in all studies (according to CLSI, 2008 - *Clinical and Laboratory Standards Institute*).

All bacterial isolates were grown on Mueller-Hinton agar (Scharlau), supplemented with 5% horse or sheep blood (bioMérieux). After antibiotic disc distribution on plates, campylobacters isolates were incubated at 42°C for 48h under microaerobic conditions
(5% O_2, 10% CO_2 and 85% N_2) in gas tight containers while QC strains were incubated at 37ºC for 24h.

2.7. Mutation detection for erythromycin resistance – RFLP-PCR

Detection of the occurrence of the point mutation at position 2075 was done by RFLP-PCR, *Restriction Fragment Lenght Polymorphism – Polymerase Chain Reaction*. The *Campylobacter* strains ZIM K49/4, ZIM 140, ZIM 375-06 were used as controls (**Table 6**). *E. coli* CCUG 42744 was a used as a negative control strain.

Table 6 - Control strains used in the detection of the point mutation A2075G 23S rRNA by RFLP-PCR.

Control strain	Species	Origin	Characteristics
ZIM K49/4	*C. jejuni*	Domestic birds, Slovenia	Erythromycin susceptible (Ery^S)
ZIM 140	*C. coli*	Domestic birds, Slovenia	Erythromycin resistant (Ery^R)
ZIM 375-06	*C. jejuni*	Human	Erythromycin resistant (Ery^R)

The methodology used was performed according to Leser *et al.* (1997) and Kurinčič *et al.* (2007) with modifications, and was the same for both *C. jejuni* and *C. coli* strains. A forward, Ery23SFor (5′GTAAACGGCCGTAACTA3′) (Stabvida) and a reverse primer, Ery23SRev (5′GACCGAACTGTCTCACGACG3′) (Stabvida) were used to amplify a 714 bp long fragment. A mutation at position 2075 resulted in five fragments after BsmAI (Biolabs) digestion (DNA fragments attained: 311, 226, 102, 57 and 18 bp). The amplification was carried out in the following conditions: an initial denaturation step at 95ºC for 5 min, followed by 35 cycles of denaturation at 95ºC for 1min, annealing at 51.9ºC for 1min, and extension at 72ºC for 1min, and a final extension at 72ºC for 5 min, were performed using a Doppio thermocycler (VWR, Belgium). Digestion with BsmAI was performed for 1h at 50ºC. The fragments were separated at 50 volts for 1h (GelXL plus, Labnet) on 1.5% agarose (SeaKem) gel.

2.8. Multiple resistant strain selection

From the original 258 isolated, 14 strains of each production system poultry samples, 14 strains of human origin and 24 strains of retail origin were selected concerning their resistance to more than two antibiotic classes, preferably over 3 antibiotic classes (Table 7). Selection was performed mainly according to the previously attained results for disc diffusion susceptibility testing. Selected strains were screened for tetracycline, gentamicin, erythromycin and fluoroquinolone resistance by minimum inhibitory concentrations testing. The occurrence of point mutations potentially responsible for erythromycin and fluoroquinolone resistance, respectively were determined by MAMA-PCR. For intensive production poultry origin isolates the selection was based on previous results from Borges (2009).

Table 7 - Comprehensive resume of all the isolates initially used concerning: Isolates origin, number of isolates for each origin (nA), species identification (ID), number of each species for each origin (n_B) and number of multiple resistant strains (n_C).

Strain origin	**n_A**	ID	**n_B**	**n_C**
Extensive indoor production system samples	40	*C. jejuni*	22	**8**
		C. coli	18	**6**
Organic production system samples	44	*C. jejuni*	27	**3**
		C. coli	17	**11**
Intensive production system samples	43	*C. jejuni*	17	**6**
		C. coli	26	**8**
Human patients samples	65	*C. jejuni*	45	**8**
		C. coli	20	**6**
Retail samples	66	*C. jejuni*	1	**0**
		C. coli	65	**24**
Totals	N=258	*C. jejuni*	112	**26**
		C. coli	146	**54**

2.9. Antimicrobial susceptibility testing: Minimum inhibitory concentrations

Minimum inhibitory concentrations (MICs) are considered the "gold standard" for determining the susceptibility of organisms to antimicrobials and are therefore used to judge the performance of all other methods of susceptibility testing. MIC is defined as the lowest concentration of a drug that will inhibit the visible growth of an organism after incubation

(Andrews, 2006). MICs were performed on the previously selected strains for 4 different antibiotics: ciprofloxacin, erythromycin, tetracycline and gentamicin (**Table 8**).

The choice of disinfectant molecules was limited to chloride and quaternary ammonium compounds. These disinfectant groups are widely used in the food industry being, their use, possible in the dilution agar method of MIC measurement according to Peyrat *et al.* (2007). Benzalkonium chloride (CB) and sodium hypochloride (SH) were tested on different concentrations.

Table 8 - List of all antibiotics and disinfectants used for MIC evaluation.

Biocide	Supplier	Solvents	Storage
		Antibiotics	
Ciprofloxacin	AppliChem	Hydrochloride 0,1M, pH4	4ºC, 2 weeks protected from light
Erythromycin	AppliChem	Ethanol 95%	4ºC, 1 weeks protected from light
Gentamicin	Sigma-Aldrich	Water	4ºC, 6 months protected from light
Tetracycline	Sigma-Aldrich	Water	4ºC, 1 week protected from light
		Disinfectants	
CB	Sigma-Aldrich	Water	4ºC, 3months protected from light
HS	Sigma-Aldrich	Water	4ºC, 3months protected from light

2.9.1.Preparation of biocide solutions

Preparation of biocide solutions for agar dilution susceptibility testing (Table 6) was performed according to EUCAST- ESCMID (2000) and Wiegand *et al.* (2007).

2.9.2.MIC procedure

The MIC was defined as the lowest concentration of an antimicrobial agent that inhibited visible growth completely. MIC procedure was performed according to EUCAST- ESCMID, 2000. Bacterial strains were grown on Columbia Blood agar (bioMerieux), supplemented with 5% horse blood and incubated at 42°C for 48h under microaerobic conditions (5% O_2, 10% CO_2 and 85% N_2) in gas tight containers. After growth bacterial strains were resuspended on saline steril water reaching 0.5 McFarland and spoted on Mueller-Hinton blood agar (Scharlau), supplemented with 5% horse or sheep blood (bioMerieux) and incorporated with each concentration of each antimicrobial under evaluation.

Campylobacter strains were incubated at 42°C for 48h under microaerobic conditions (5% O_2, 10% CO_2 and 85% N_2) in gas tight containers. Spotting was performed on a laminar flow class II cabinet (Telstar). *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212 and *Campylobacter jejuni* NCTC11168, for which quality control ranges have been established, were included as quality control strains (according to CLSI - *Clinical and Laboratory Standards Institute*).

Ranges for antibiotic concentration for susceptibility testing were chosen according to Payot *et al.* (2002), Payot *et al.* (2004), Peyrat *et al.*(2007), CA-SFM (2010), (**table 9**). Ranges for disinfectant concentration susceptibility testing were chosen according to Peyrat *et al.* (2007) with modifications. The breakpoints adopted for MIC interpretation are presented on **Table 10**, according to CA-SFM (2010).

Table 9 - Comprehensive resume concerning the range of antibiotic and disinfectant concentrations under evaluation (E, erythromycin; CIP, ciprofloxacin; GM, gentamicin; TE, tetracycline; BC, benzalkonium chloride and HS, sodium hipochloride). Grey areas represent range concentrations for which MICs were performed. White areas represent concentrations not tested by MIC in the present work.

µg/mL	0.03	0.06	0.125	0.250	0.5	1	2	4	8	16	32	64	128	256	512
Antibiotics															
E															
CIP															
GM															
TE															
Disinfectants															
BC															
SH															

Table 10 – Breakpoints for MIC interpretation according to CA-SFM, 2010

Antibiotics	Breakpoints mg/L		
	S	**I**	**R**
Gentamicin	≤2	2-4	>4
Cirpofloxacin	≤0.5	0.5-1	>1
Erythromycin	≤1	1-4	>4
Tetracycline	≤4	4-8	>8

2.10. Efflux pump: the efflux pump inhibitor

The efflux system CmeABC is a key player for intrinsic antibiotic resistance and is also required for the acquired resistance to fluoroquinolones. In order to determine the effect of this efflux pump in ciprofloxacin resistance different concentrations of an efflux pump inhibitor Phe-Arg-β-aphthylamide, EPI – PAβN was incorporated alongside the different ciprofloxacin concentrations previously mentioned on Mueller-Hinton blood agar (Scharlau), supplemented with 5% horse or sheep blood. MICs were determined in the presence of the EPI Phe-Arg-β-naphthylamide (Sigma) at the following concentrations: 10, 20, 40, 60, and 120 mg/ml according to Payot *et al.* (2004).

2.11. Mismatch Amplification Mutation Assay-Polymerase Chain Reaction

In order to determine the presence or absence of the point mutations attributed to high- level erythromycin and ciprofloxacin resistance in both *C. jejuni* (n=26) and *C. coli* (n=54) an alternative inexpensive, rapid, accurate protocol to nucleotide sequencing was introduced: Mismatch Amplification Mutation Assay - Polymerase Chain Reaction (MAMA-PCR). This PCR technique involves the use of a conserved forward primer and a reverse mutation detection primer to generate a PCR product that will be a positive indication of the presence of the target mutations.

2.11.1. MAMA-PCR for erythromycin resistance point mutations

MAMA-PCR assay proved to be a fast and easy method to detect the presence of the point mutations in the 23S rRNA genes to which high-level resistance to erythromycin has been attributed. The methodology, adopted from Alonso *et al.*(2005) with modifications, was the same for both *C. jejuni* (n=26) and *C. coli* (n=54) strains. One conserved forward primer, 23S rRNA–F (5'TTAGCTATGTTGCCCGTACCG3') was used in conjunction with the reverse mutation primer ERY2075-R (5'TAGTAAAGGTCCACGGGGTCG3') to detect A2075G mutation, and in parallel with ERY2074-R (5'AGTAAAGGTCCACGGGGTCTGG3') to detect A2074C mutation. In both reactions, an amplicon of 485 bp was expected as a positive indication of the occurrence of the point mutations. A positive PCR control from 16S rRNA gene of *C. jejuni* and *C. coli* was used in this MAMA-PCR assay that generated an 857 bp amplicon.

The amplification was carried out in the following conditions: An initial denaturation step at 94ºC for 5 min, followed by 30 cycles of denaturation at 94ºC for 30 s, annealing at 59ºC for 30 s, and extension at 72ºC for 45 s, and a final extension at 72ºC for 5 min, were performed using a Doppio thermocycler (VWR, Belgium). The amplified PCR fragments were separated by electrophoresis at 80 volts for 50 minutes (VWR) on 1.5% agarose (SeaKem) gel and visualization was possible through LISCAO, ImageMaster (Pharmacia Biotech).

Because no positive control strains were available several strains were screened for the presence of the point mutation A2075G. One strain carrying the mutation was selected, respective PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA) was then sequenced (Stabvida): PC^{ery}. *C. jejuni* NCTC 11168 was used as a negative control strain.

2.11.2. MAMA-PCR for ciprofloxacin resistance point mutations

The protocol used for detection of mutations in the *gyrA* gene was derived from the method described by Zirnstein *et al.* (1999) and Payot *et al.* (2002) with modifications. A conserved forward primer CampyMAMAgyrA1 (5'TTAGCTATGTTGCCCGTACCG3') was used for both *C. jejuni* and *C. coli* but different reverse mutation detection primers were used for either strains: while CampyMAMA *gyrA5* (5'TTAGCTATGTTGCCCGTACCG3') was used for the detection of the potential point mutation in *C. jejuni* Campygyrmut (5'TTAGCTATGTTGCCCGTACCG3') was used for *C. coli*. A conserved, forward primer and the reverse mutation detection primers, were used together to generate a 265-bp PCR product that was a positive indication of the presence of the corresponding Thr86-to-Ile change in the *C. jejuni* GyrA.

The amplification conditions were the same for both species, except for the annealing step, and was carried out in the following conditions: initial denaturation step at 94ºC for 5 min, followed by 30 cycles of denaturation at 94ºC for 30 s, annealing at 52ºC for 30 s for *C. jejuni* and 50ºC for *C. coli*, and extension at 72ºC for 45 s, and a final extension at 72ºC for 5 min, were performed performed using a Doppio thermocycler (VWR, Belgium). The amplified PCR fragments were separated by electrophoresis at 80volts for 50 minutes (VWR) on 1.5% agarose (SeaKem) gel.

Because neither *C. jejuni* nor *C. coli* positive control strains were available, several strains were screened for the presence of the point mutation in the *gyrA* gene. One strain of each species carrying the mutation was selected. Respective PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA) and sequenced (Stabvida): PC^{gyrA}C.j and PC^{gyrA}C.c. *C. jejuni* NCTC 11168 was used as a negative control strain.

3. Results and discussion

According to EFSA, campylobacteriosis has been the most commonly reported zoonosis, since 2005. Portugal is yet to include itself on the EFSA's European Union Member States (EU MSs) that notifies yearly occurring human campylobacteriosis cases. Although several articles have been published concerning *Campylobacter* infection in humans, in Portugal much is still to be done (Cabrita *et al.*, 1992; CSSSC, 2002; Vicente *et al.*, 2008) Portugal is, however, included in the MS countries that do provide data concerning the prevalence of *Campylobacter* strains on broiler batches and the subsequent prevalence of this bacteria on foodstuffs.

The knowledge that *Campylobacter* spp., especially *C. jejuni* and *C. coli,* are considered to be the most frequent cause of human bacterial enteritis worldwide and that poultry meat is an important foodborne source, where campylobacters linger from broiler flocks till processed retail products, are now facts. Significant antibiotic resistance levels in *Campylobacter* spp. from human and animal sources has been reported and there is a growing, pressing need to better understand *Campylobacter* spp. antimicrobial resistance patterns and the reason they occur, in order to find alternative ways of controlling the prevalence of this bacteria in foodstuffs, namely in poultry meat and in humans (Mena *et al.*, 2008, Vicente *et al.*, 2008; ESFA journal, 2010).

Sampling procedure was performed in order to attain as much strain differentiation as possible.

3.1. Characterization of *Campylobacter* isolates for antibiotic resistance

The relative contribution of each isolate origin to the final number of disc susceptibility tested isolates (N=258) was analyzed (**Table 11**). Over two thirds, 75%, of the tested isolates were of poultry meat origin while the remaining isolates, 25%, were of human origin, campylobacteriosis patients.

Table 11 - Comprehensive resume of all the isolates initially used concerning: Isolates origin, number of isolates for each origin (nA), species identification and number of each species for each origin (n_B).

Isolate origin	n_A (nº of isolates) (%)	Species	n_B (nº of each species)
Extensive indoor production system samples	40 (16%)	*C. jejuni*	**22**
		C. coli	**18**
Organic production system samples	44 (17%)	*C. jejuni*	**27**
		C. coli	**17**
Intensive production system samples	43 (17%)	*C. jejuni*	**17**
		C. coli	**26**
Human patients samples	65 (25%)	*C. jejuni*	**45**
		C. coli	**20**
Retail samples	66 (25%)	*C. jejuni*	**1**
		C. coli	**65**
Totals	258	*C. jejuni*	**112**
		C. coli	**146**

From the initial 258 strains, 43% were identified as *Campylobacter jejuni* (n=112) while 57% were *Campylobacter coli* strains (n=146) (**Figure 3**). According to Mena *et al.* (2008), Saenz *et al.* (2000) and Fraqueza, (2009), a higher occurrence of *C. jejuni* has been associated with poultry products, although such a tendency was not observed in this study. From the 193 isolates from poultry meat products used, *C. coli* isolates accounted for 65% against the 35% for *C. jejuni*. Careful observation indicates that such results are due to *C.coli* considerably higher representativeness in retail samples, which overwhelms the relatively similar

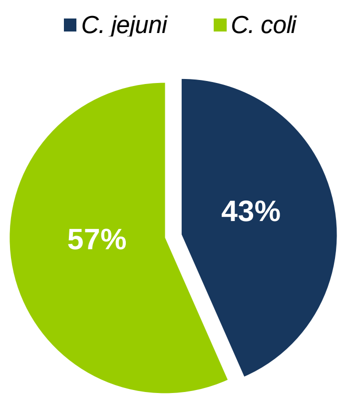

Figure 3 – Relative contribution of each identified *Campylobacter* species for the total number of isolates (N=258).

representativeness of both species for the other three origins. For human isolates there seems to also be an expected predominance of *C. jejuni*, as reported by Saenz *et al.* (2000) and this was, in fact, the case: 69% *versus* 31% for *C. coli*.

3.2. Extensive indoor production system samples origin strains

From the total initial *Campylobacter* spp. isolates (N=258), 16% were from broiler samples of extensive indoor production system origin, n=40, (Table 11). After identification, 55% were attributed to *C. jejuni* (n=22, out of the 40 total extensive indoor isolates) while 45% were identified as *C. coli* (n=18, out of the total 40 extensive indoor isolates).

In view of the results for disc susceptibility testing obtained for extensive indoor production system origin strains regarding each of the 11 antibiotics tested one can observe that β-lactams, (ampicilin and amoxacilin+clavulanic acid) alongside the fluoroquinolones, (nalidixic acid, ciprofloxacin, norfloxacin and ofloxacin) showed the highest levels of resistance, respectively: 70% (n=28), 53% (n=21), 83% (n=33), 75% (n=30), 68% (n=27) and 58% (n=23) (**Figure 4**). Resistance levels for these antibiotics were all over 50% specially for both nalidixic acid and ciprofloxacin. Strains showed the highest susceptibility, 93% (n=37) to chloramphenicol. Similar resistant and susceptibility ratios were attained for tetracyclin, 38% resistance (n=15) and gentamicin, 45% resistance (n=18) which was also the result for trimetropim/sulphametoxazole. For the macrolide erythromycin, 30% (n=12) of the tested were resistant. Resistance to this antibiotic has been mainly attributed to the occurrence of point mutation at position 2075 in the 23S rRNA. Of the 12 (n=12) strains that tested resistant for erythromycin, 8 isolates were corroborated by the detection of the point mutation by RFLP-PCR. For the remaining strains, according to Kurinčič *et al.* (2007), absence of corroboration may be due to the occurrence of alternative mutations occuring at position 2074 in the 23S rRNA.

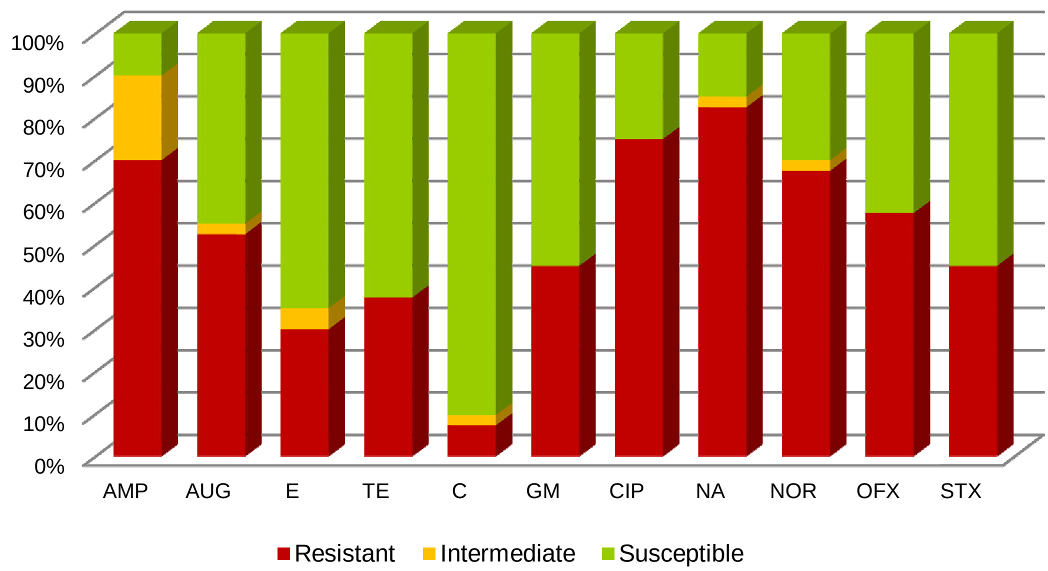

Figure 4 – Frequency of resistant to susceptible isolates of Campylobacter from extensive indoor production system origin isolates after perfoming disc diffusion testing. AMP, ampicilin; AUG, amoxacilin/clavulanic acid; E, erythromycin; TE, tetracycline; C, chloramphenicol; GM, gentamicin; CIP, ciprofloxacin; NA, nalidixic acid; NOR, norfloxacin; OFX, ofloxacin; STX, Trimethoprim/sulfamethoxazole.

Disc diffusion results revealed that only one strain was susceptible to all classes of tested antibiotics (**Figure 5**). Considering the definition of multiple resistance presented by SCENIHR (2009) stating that multiple resistance (MR) occurs when a bacterial strain is resistant to several, different antimicrobials or antimicrobial classes, we considered that multiple resistance occurs when resistance is found for more than 2 classes. The majority of isolates, 35% (n=14) were found to be resistant to a maximum of 4 classes of antibiotics, 5% (n=2) were resistant to 5 classes of antibiotics and 8% (n=3) were resistant to 6 of the 7 classes tested. It is then safe to conclude that nearly half, 48% (n=19), of the tested isolates were multiple-resistant strains. No strain was found to be resistant to all 7 antibiotic classes under evaluation.

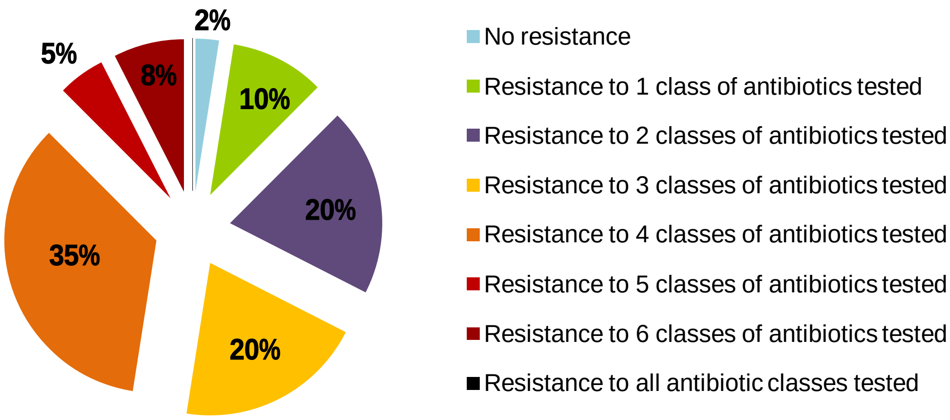

Figure 5 - Extensive indoor production system origin isolates resistance to different antibiotic classes, identification of multiple drug resistant strains.

Although no particularly staggering difference was observed between species, it is possible to scrutinize (**Table 12**) that *C. coli* isolates showed less resistance levels than its counterpart with the exception of tetracycline and norfloxacin. Considering *C. jejuni*'s resistance in broiler meat it was observed that, for the isolates in this study, resistance patterns were considerably higher for gentamicin, and erythromycin then those reported by the EFSA (2010) on antimicrobial resistance in zoonotic and indicator bacteria from animals and food in the European Union in 2008. For gentamicin, a resistance level for *C. jejuni* of 13% against the 55% resistant isolates was observed in this study. A considerable discrepency was also observed for erythromycin for which, instead of the 6% EFSA reported, resistance levels reached 32% of the tested isolates. Reported resistance levels for ciprofloxacin and nalidixic acid ranged from 31-100%, for these antibiotics resistance isolates settled for, respectively 86% and 82%. For tetracycline resistance levels attained in this study were quite similar as the reported ones, 32% to 38%.

Table 12 – Summary of the results attained for the 11 tested antibiotics for *C. jejuni* and *C. coli* for extensive indoor production system isolates. n_R, number of tested resistant strains for the mentioned antibiotics.

Antibiotic	***C. jejuni*** (N=22)		***C. coli*** (N=18)	
	n_R/N	**(%)**	**n_R/N**	**(%)**
Ampicilin	18/22	82%	10/18	56%
Amoxacilin	14/22	64%	7/18	39%
Erythromycin	7/22	32%	5/18	28%
Tetracycline	7/22	32%	8/18	44%
Chloramphenicol	2/22	9%	1/18	6%
Gentamicin	12/22	55%	6/18	33%
Ciprofloxacin	18/22	82%	12/18	67%
Nalidixic acid	19/22	86%	14/18	78%
Norfloxacin	14/22	64%	13/18	72%
Ofloxacin	13/22	59%	10/18	56%
Trimethoprim/sulfamethoxazole	11/22	50%	7/18	39%

Caecum and carcass (breast skin) samples takes a representation of 23% (n=9) and 30% (n=12) respectively while breast meat has a 48% (n=19) contribution. No particular distinction, concerning antibiotic resistance, was observed, between strains, isolated from these different samples of extensive indoor production system.

3.3. Organic production system samples origin strains

From the total initial *Campylobacter* spp. strains (N=258), 17% were from broiler samples of organic production system origin (n=44, out of 258 total strains) (Table 11). 61% were attributed to *C. jejuni* (n=27, out of the 44 total organic strains) while 39% were *C. coli* (n=17, out of the total 44 organic strains).

Disc susceptibility testing for organic production system origin strains, regarding each of the 11 antibiotics tested revealed that most isolates were resistant to the fluoroquinoles used (**Figure 6**): Ciprofloxacin, 89%; Nalidixic acid, 91%; Norfloxacin, 89% and Ofloxacin, 84%. A previous study performed for isolates from organic production samples in the United States revealed the same, understandable paralelism between quinolone results although they came across much lower resistance patterns, 1% (Luangtongkum *et al.*, 2006). The β-lactam ampicilin, as well as tetracycline had 73% resistant isolates (n=32) against the 5% and 52%, respectively, observed in the previously mentioned study. There was a considerable difference between the number of resistant strains for ampicilin (n=32) in comparisson to the number of resistant strains for amoxacilin/clavulanic acid, (n=19).

Antibiotic resistance evaluation by disc diffusion testing was performed and the response of both species to the 11 antimicrobials tested. Regarding the general panorama, no greater difference was observed between species, *C. coli* showed higher levels of resistance when compared to *C. jejuni* with the exception of gentamicin (**Table 13**).

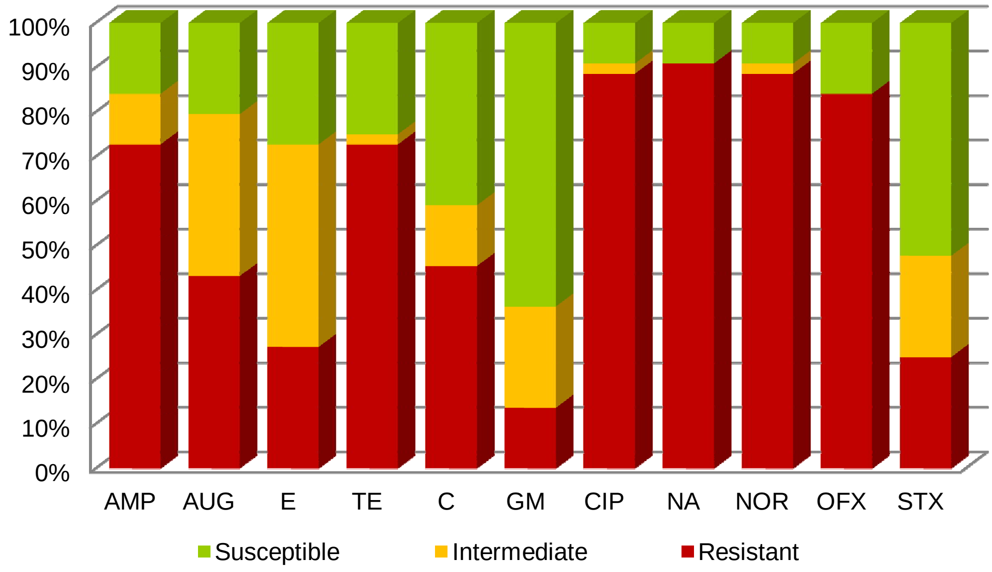

Figure 6 - Disc susceptibility testing for organic production system origin isolates. AMP, ampicilin; AUG, amoxacilin/clavulanic acid; E, erythromycin; TE, tetracycline; C, chloramphenicol; GM, gentamicin; CIP, ciprofloxacin; NA, nalidixic acid; NOR, norfloxacin; OFX, ofloxacin; STX, Trimethoprim/sulfamethoxazole.

C. coli isolates were particularly resistant to fluoroquinolones raging between 94%, for both ofloxacin and norfloxacin, to 100% for nalidixic acid and ciprofloxacin while *C. jejuni* resistance levels ranged from 70% for ofloxacin till 78% for nalidixic acid. Chloramphenicol had susceptibility levels of 41% (n=20), while 52% (n=23) of strains were trimethoprim/sulfamethoxazole susceptible. Gentamicin had the lowest resistance level, given that only 14% of tested strains were resistant. For erythromycin the exact same number of strains, n=12, were found to be resistant and susceptible while almost half, 46%, of the isolates were classified as intermediate. Of the 12 (n=12) strains that tested resistant for erythromycin, 12 (n=12) were corroborated by the detection of the point muation by RFLP-PCR.

Table 13 - Summary of the **r**esults attained for the 11 tested antibiotics for *C. jejuni* and *C. coli* for organic production system isolates.

Antibiotic	***C. jejuni*** (N=27)		***C. coli*** (N=17)	
	n_R/N	**(%)**	**n_R/N**	**(%)**
Ampicilin	18/27	67%	14/17	82%
Amoxacilin	9/27	33%	10/17	59%
Erythromycin	5/27	19%	7/17	41%
Tetracycline	17/27	63%	14/17	82%
Chloramphenicol	11/27	41%	8/17	47%
Gentamicin	5/27	19%	1/17	6%
Ciprofloxacin	20/27	74%	17/17	100%
Nalidixic acid	21/27	78%	17/17	100%
Norfloxacin	20/27	74%	16/17	94%
Ofloxacin	19/27	70%	16/17	94%
Trimethoprim/sulfamethoxazole	6/27	22%	5/17	29%

According to Luangtongkum *et al.* (2006), a considerable decrease of antimicrobial resistance should be observed considering the practices performed for raising organic poultry. Isolates in the present study went against such observations. Rodenburg *et al.* (2004), stated that although organic poultry is generally assumed to be safer and healthier, the fact that these animals have access to an outdoor run allows them to contact with wild birds, rodents, manure and various other *Campylobacter* resistant strain sources enabling the transmission of resistant strains to organic poultry infecting the entire flocks.

Disc diffusion testing revealed that only one isolate was susceptible to all classes of antibiotics tested (**Figure 7**). The majority of strains, 27% (n=12) were found to be resistant to a maximum of 4 classes of antibiotics, 14% (n=6) were resistant to 5 classes of antibiotics and 11% (n=5) were resistant to 6 of the 7 classes tested. Over half the strains analyzed, 75% (n=33), of the tested strains were multiple-resistant strains. No strain was found to be resistant to all 7 antibiotic classes under evaluation. Luangtongkum *et al.* (2006) also screened strains for multiple resistance concluding that, for broiler isolates of organic production system, only 7% of all tested strains showed to resist to 4 different antibiotic classes considerably less than the 27% observed in this study.

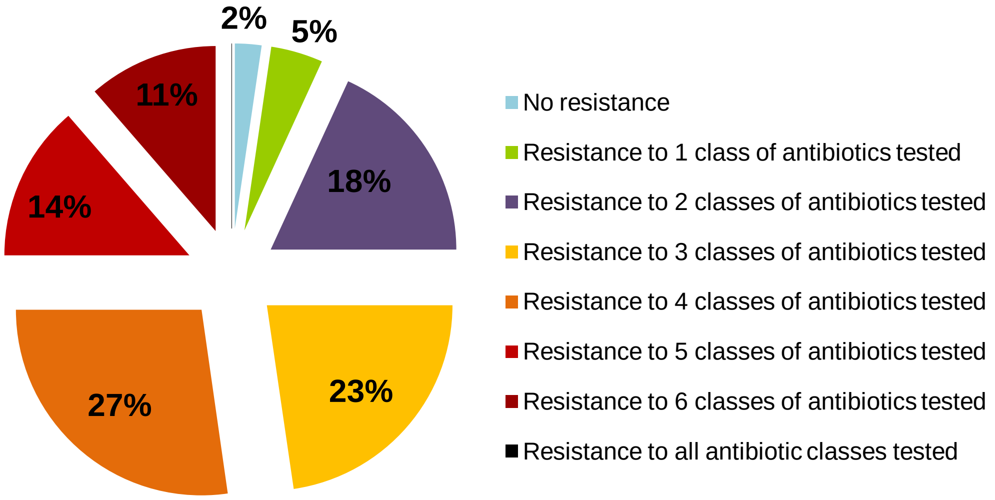

Figure 7 - Organic production system origin strains resistance to different antibiotic classes. Identification of multiple drug resistant strains.

Caecum, carcass (breast skin) and breast meat samples took a representation of 21% (n=9); 25% (n=11) and of 46% (n=24) respectively. No particular distinction, concerning antibiotic resistance, was observed between strains isolated from these different samples of organic production system although carcass (breast skin) samples showed a higher number of strains resistant to 2 of the classes of antibiotics evaluated, while caecum and breast meat showed multiple resistances, to 3 and 4 classes respectively.

3.4. Intensive production system samples origin strains

Intensive production system samples had a contribution of 16% (n=43 out of the 258 total strains) isolates. *C. jejuni* accounted for 40% (n=17, out of the 43 total intensive strains) while 61% were identified as *C. coli* (n=26, out of the total 43 intensive strains).

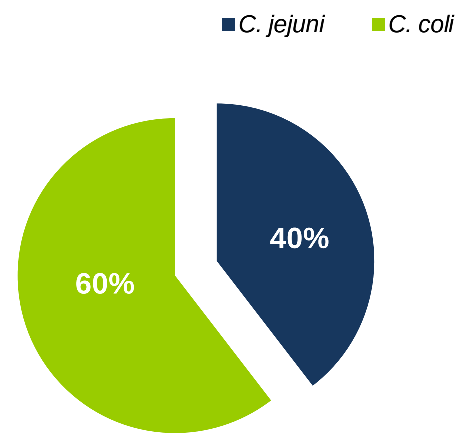

Figure 8 - Relative contribution of each identified *Campylobacter* species for the total number of intensive production systems.

In opposition to what was previously described by Mena *et al.* (2004), there was a higher occurrence of *C. coli* in comparison to *C. jejuni* for these isolates (**Figure 8**). Such a situation may be due to the restriction of sample size, questioning the representativeness of the data.

3.5. Human stool samples origin strains

From the total initial *Campylobacter* spp. strains (N=258), 25% were from human stool sample origin (n=65, out of 258 total strains) (**Table 11**).

Stool sample origin strains were attained from 2008 till 2010 from patients from different Portuguese hospitals. More male isolates, 62%, than female, 38%, (male/female ratio = 1.6) were attained except for young adults, these were also the observations published by Gallay *et al.*, 2007 who observed a male/female ratio of 1.2 with the exception of young adults (**Figure 9**). The distribution of *Campylobacter* spp isolates according to the gender and age of the patient revealed that the ages from 0 to 4 years were particularly affected as well as for adults from ages 15 till 54. It is worth considering the hypothesis that newborns and infants raise more concern to parents or caretakers and hence the increase amount of detection for this particular age group.

Strain identification had been performed at INSA: 69% were *C. jejuni* (n=45, out of the 65 total human strains) while 31% were identified as *C. coli* (n=20, out of the total 65 human strains).

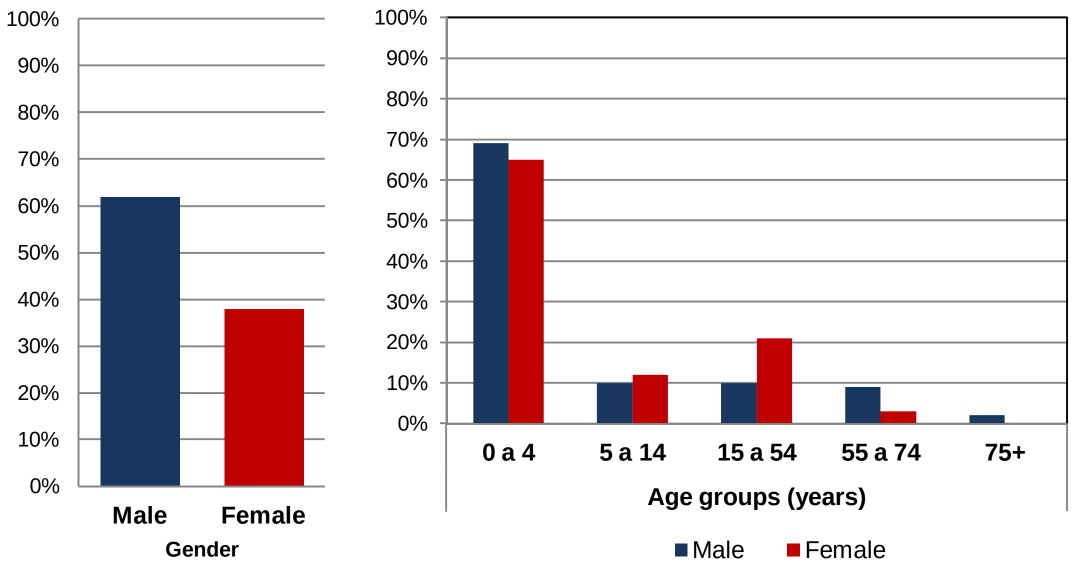

Figure 9 – Gender related Campylobacter spp. isolated from stool samples and the distribution of *Campylobacter* spp. isolates according to age and gender of patient, Portugal, 2008 – 2010.

Disc diffusion testing made it possible to compare antibiotic resistance shown by both species in comparison to each other for the 11 antimicrobials tested (**Table 14**). Both species were entirely susceptible to chloramphenicol. Similarity between strains was also attained for ampicilin, 73% and 75% for *C. jejuni* and *C. coli* respectively, which was not what Gallay *et al.* (2004) found. In Gallay *et al.* (2004) study, ampicilin resistant level was higher than those observed in this study and were higher for *C. jejuni* (38%) in comparison to *C. coli* (15%). For fluoroquinolones, it was found in the present study that *C. jejuni* strains were tendencially more resistant then *C. coli* strains. For ciprofloxacin 84% *C. jejuni* isolates were resistant while 60% of *C. coli* isolates were resistant strains.

Table 14 - Summary of the results attained for the 11 tested antibiotics for *C. jejuni* and *C. coli* for human stool sample isolates.

Antibiotic	***C. jejuni*** (N=45)		***C. coli*** (N=20)	
	n_R/N	**(%)**	**n_R/N**	**(%)**
Ampicilin	33/45	73%	15/20	75%
Amoxacilin/clavulanic acid	18/45	40%	16/20	80%
Erythromycin	8/45	18%	13/20	65%
Tetracycline	23/45	51%	1/20	5%
Chloramphenicol	0/45	0%	0/20	0%
Gentamicin	4/45	9%	8/20	40%
Ciprofloxacin	38/45	84%	12/20	60%
Nalidixic acid	38/45	84%	16/20	80%
Norfloxacin	31/45	69%	15/20	75%
Ofloxacin	37/45	82%	16/20	80%
Trimethoprim/sulfamethoxazole	28/45	62%	15/20	75%

In Gallay *et al.* (2004) study the reverse occurred and much lower level of ciprofloxacin resistance were reported, with *C. coli* having higher levels of ciprofloxacin resistance, 42% against *C. jejuni*, 25%. On the one hand, results provided by Gallay *et al.* (2004) pertain to human isolates from France and reported fluoroquinolone considerably lower resistance levels than those obtained in this study. On the other hand, the results attained in the present study approached the results provided by Vicente *et al.* (2008) that revealed an 81% resistant level to the fluoroquinolone ciprofloxacin. It is also worth mentioning the difference found for erythromycin. Engberg *et al.* (2001) reported that typically higher frequency of erythromycin resistance was found in *C. coli* than in *C. jejuni*. In this study, while 18% of *C. jejuni* strains were found to be resistant to this antibiotic against 65% of *C. coli* strains. A similar patter, although for lower levels of erythromycin resistance, was observed by Gallay *et al.* (2004): *C. jejuni* had a resistant level of only 1% to erythromycin, 13% *C. coli* isolates were erythromycin resistant. A similar but reverse occurrence can be observed for tetracycline, while this time *C. coli* had only 1 strain resistant to tetracycline (5%) more than half of *C. jejuni* strains, 51%, had the resistant phenotype. Gallay *et al.* (2004) reported the opposite: 53% of *C. coli* isolates were resistant against the 29% *C. jejuni* resistant isolates. Also, particularly higher levels of gentamicin resistance were registered for *C. coli* human strains. It is worth adding that for most antibiotics, with the exception of tetracycline and gentamicin, which are included in classes that share the same bacterial primary target, *C. coli* had similar to higher levels of resistance when compared to *C. jejuni*.

According to the results from disc susceptibility testing obtained for stool samples of human campylobacteriosis patients origin strains, regarding each of the 11 antibiotics tested one can observe that flouroquinolones had the highest level of resistance ranging from 71% for norfloxacin (n=46) to 83% for nalidixic acid (n=54). 77% (n=50) strains were resistant to ciprofloxacin and 82% were ofloxacin resistant, (n=53) (**Figure 10**). Vicent *et al.* (2008), came across a similar result concedering ciprofloxacin resistance: 80.5%.Comparatively, also the β-lactam ampicilin had relatively high levels of resistance, up to 74% (n=48) of human origin strains were ampicilin resistant while for amoxacilin/clavulanic acid resistance was as high as 52%. For tetracycline and gentamicin results were quite uneven, 37% (n=24) and 19% (n=12) respectively. 34% (n=22) of tested human strains were susceptible to trimethoprim/sulfamethoxazole which was similar to erythromycin, 32% resistant isolates. To chloramphenicol strains were entirely, 100% (n=0) susceptible.Of the 21 (n=21) strains that tested resistant for erythromycin only 11 (n=11) were corroborated by the detection of the point mutation by RFLP-PCR. For the remaining strains, according to Kurinčič *et al.* (2007) absence of corroboration may be due to the occurrence of alternative mutations

Antibiotic disc diffusion protocol performed for human strains made it possible to draw conclusions considering the occurrence of bacterial strains resistant to several, different antimicrobial classes (**Figure 11**). Multiple resistance occurred for 75% of human strains (n=49). Although no strains were found to be entirely susceptible none were also found to be resistant to more than 5 classes of antibiotics.

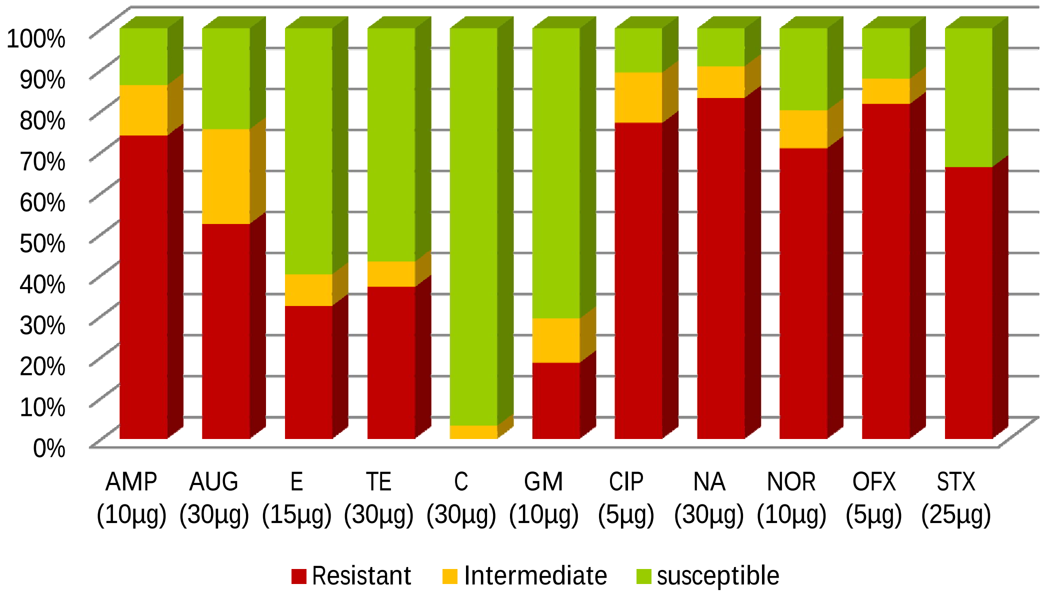

Figure 10 - Disc susceptibility testing for human stool origin strains. AMP, ampicilin; AUG, amoxacilin/clavulanic acid; E, erythromycin; TE, tetracycline; C, chloramphenicol; GM, gentamicin; CIP, ciprofloxacin; NA, nalidixic acid; NOR, norfloxacin; OFX, ofloxacin; STX, Trimethoprim/sulfamethoxazole.

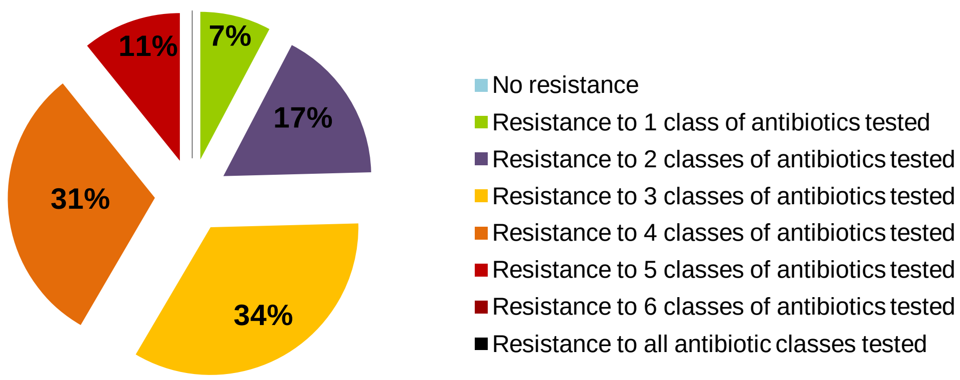

Figure 11 - Extensive indoor production system origin isolates resistance to different antibiotic classes, identification of multiple drug resistant strains.

The majority of strains were resistant to a maximum of 3 antibiotic classes, 34%. Multiple resistances in humans is a serious matter due to the fact that infections in humans reflect both foodstuffs, mainly poultry meat, contamination with multiple drug resistance strains and the potential hazard of fail on good practice utilization of antibiotic at animal production level and human prescription and consumption. Gallay *et al.* (2004) reported that 58% of *Campylobacter* isolates were resistant to 1 or more drugs. This number is approximately half of what we attained in the present study. It was also reported by Gallay *et al.* (2004) that 20% were resistant to 3 or more antibiotics while in the present study 20% accounts for little less than one third of isolates resistant to 3 or more antibiotics.

3.6. Retail origin strains

From the total initial *Campylobacter* spp. strains (N=258), 25% were from retail origin (n=65, out of 258 total strains) (**Table 11**). After species identification 2% were attributed to *C. jejuni* (n=1, out of the 66 total retail strains) while 99% were identified as *C. coli* (n=65, out of the total 66 retail strains). *C. jejuni* contribution was discarded at this point for further purposes.

Disc diffusion method for the 11 antimicrobials tested was performed (**Figure 12**). Considering the absence of *C. jejuni* strains disc diffusion test results were performed for *C. coli*. Similar high resistance patterns were observed between the fluoroquinolones: nalidixic acid, 91% (n=59); ciprofloxacin, 92% (n=60); norfloxacin, 91% (n=60) and ofloxacin, 91% (n=59), these resistance levels were higher than those reported by Ge *et al.* (2003) and Kurinčič *et al.* (2005), who observed 41% and 35%, and 59% and 76% resistant isolates to respectively nalidixic acid and ciprofloxacin. Mena *et al.* (2004) did find fluoroquinolone resistant patterns that best resembled those attained in this study. The β-lactams, ampicilin and amoxacilin/clavulanic acid had similar resistance patterns, respectively, 49% (n=32) and 39%(n=25). Staggering was the difference between the results for tetracyclin, 91% (n=59) and gentamicin, 6% (n=4) due to the fact that both antimicrobial share primary targets. *C. coli* was almost entirely susceptible to chloramphenicol showing a 95% (n=62) susceptibility to the phenicol. Of the 27 strains that tested resistant for erythromycin 15 were corroborated by the detection of the point mutation by RFLP-PCR. This correspondes to a lower number of erythromycin resistant isolates than the 54% reported by Ge *et al.* (2003). One of the tested strains was phenotypically intermediate although it carried the mutation.

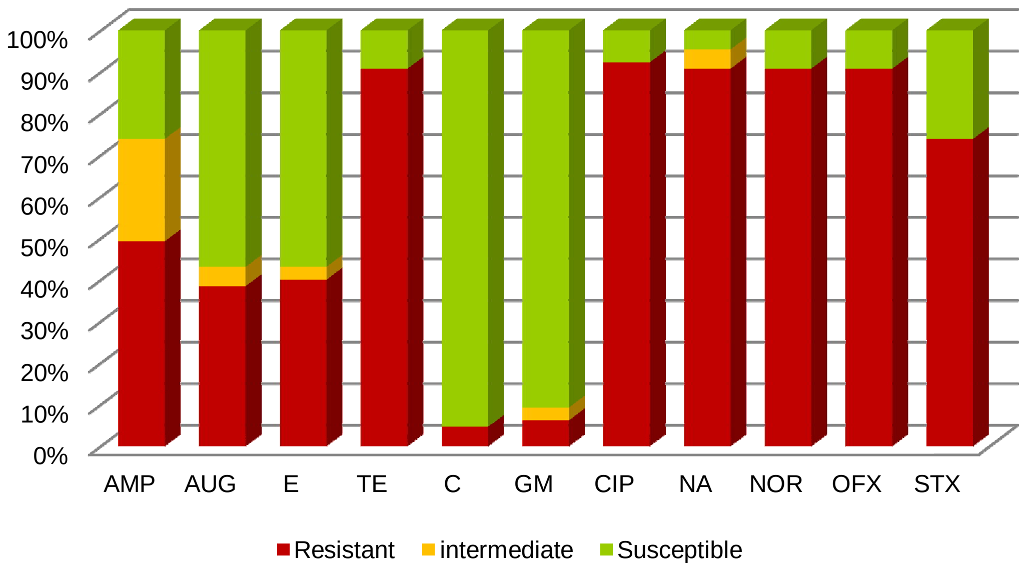

Figure 12 - Disc susceptibility testing for processed poultry retail products. AMP, ampicilin; AUG, amoxacilin/clavulanic acid; E, erythromycin; TE, tetracycline; C, chloramphenicol; GM, gentamicin; CIP, ciprofloxacin; NA, nalidixic acid; NOR, norfloxacin; OFX, ofloxacin; STX, Trimethoprim/sulfamethoxazole.

Results considering the occurrence of bacterial strains resistant to several different antimicrobial classes: multiple resistance, were acquired by performing antibiotic disc diffusion protocol for retail strains (**Figure 13**). Multiple resistance occurred for 83% of retail strains (n=54). No strains were found to be entirely susceptible to every class of antimicrobials tested. 20% (n=13) of tested strains were found to be resistant to more than 5 classes of antibiotics. The majority of strains were resistant to a maximum of 4 antibiotic classes, 37%. Resistance to the whole panoply of tested antibiotics, 7 distinct classes, was found for 2 strains (n=2), 3%. One might assume that such observations may be due to the fact that such isolates are subjected to various types of meat products preservatives and therefore developing cross-resistance to antibiotics.

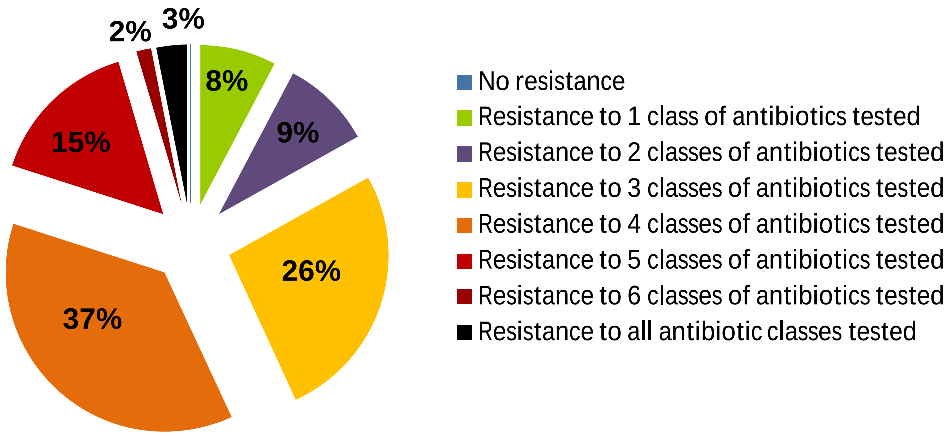

Figure 13 - Processed poultry retail products resistance to different antibiotic classes, identification of multiple drug resistant strains.

At a glance, results concerning broiler, human and retail samples strain resistance to 7 different classes of antibiotics are summarized in **Tables 15 and 16**.

Table 15 – Comprehensive resume for antibiotic resistance to the 7 tested antibiotic classes for ***C. jejuni*** isolates from broiler, human and retail origin. ND, not determined.

	AMP		E		TE		C		GM		CIP		STX	
	n_R/N	%R	n_R/N	%R	n_R/N	%R	n_R/N	%R	n_R/N	%R	n_R/N	%R	n_R/N	%R
EIPS	18/22	82	7/22	32	7/22	32	2/22	9	12/22	55	18/22	82	11/22	50
OPS	18/27	67	5/27	19	17/27	63	11/27	41	5/27	19	20/27	74	6/27	22
HS	33/45	73	8/45	18	23/45	51	0/45	0	4/45	9	38/45	84	28/45	62
R	**ND**													

According to EFSA report (2010) on antimicrobial resistance in zoonotic and indicator bacteria from animals and food in the European Union in 2008 for *C. jejuni* isolates from broiler meat provided by six MSs revealed that resistance to tetracycline was 38% for the reporting MS group, with the highest occurrence in Portugal (73%). This work tested strains that showed resistance levels of 63% for organic strains. EFSAs reported resistance levels to erythromycin and gentamicin of 6% and 13%, respectively, this work tested strains had resistance levels of 32% and 55%. The resistance levels for ciprofloxacin at reporting MS group level was 46% compared with 82% attained in this work. Although for some of the reposting countries ciprofloxacin resistance varied between 31% and 100%. Attained results, although staggering alarming when compared with the DANMAP, (2009), do actually generally comply with the observations of Sáenz *et al.* (2000) for Spanish strains or Petozzi *et al.* (2003) for northeastern Italy. This situation agrees with the already identified with the different geographical Campylobacter spp. isolation/ resistance patterns throughout Europe where northern countries appear less afflicted than their southern counterparts.

Table 16 - Comprehensive resume for antibiotic resistance to the 7 tested antibiotic classes for ***C. coli*** isolates from broiler, human and retail origin

	AMP		E		TE		C		GM		CIP		STX	
	n_R/N	%R	n_R/N	%R	n_R/N	%R	n_R/N	%R	n_R/N	%R	n_R/N	%R	n_R/N	%R
EIPS	10/18	56	5/18	28	8/18	44	1/18	6	6/18	33	12/18	67	7/18	39
OPS	14/17	82	7/17	41	14/17	82	8/17	47	1/17	6	17/17	100	5/17	29
HS	15/20	75	13/20	65	1/20	5	0/20	0	8/20	4	12/20	60	15/20	75
R	32/65	49	26/65	40	59/65	91	3/65	5	4/65	6	60/65	92	48/65	74

Regarding the general panorama for isolates susceptibility testing of broiler meat products of extensive indoor production and organic production systems and retail processed products, there seemed to be a slight tendency for extensive indoor isolates to have a higher susceptibility to the different antibiotics tested in comparison to organic production systems. Such observation was stronger, regarding results for the fluoroquinolone, ciprofloxacin for which 89% of organic isolates were resistance against the 75% resistant isolates detected for extensive indoor isolates. Such tendency was even more evident for tetracycline for which 37.5% of extensive indoor isolates were found to be resistant against the 73% of isolates. For macrolide resistance the opposite was regarded as, 27% of organic isolates were resistant *versus* 75% for extensive indoor production. Ciprofloxacin and tetracycline resistance was higher for retail processed poultry reaching resistance levels of 92% and 91% respectively. For human strains a 77% fluoroquinolone resistance was observed which is relatively close to what was described by Vicente *et al.* (2008) and also by Engberg *et al.* (2001) for Spanish resistant isolated strains. Engberg *et al.* (2001) also stated that in these regions, where quinolone resistance is highly endemic and *Campylobacter* spp. predominate; fluoroquinolones should not be recommended for community-acquired bacterial diarrhea.

3.7. Effect on ciprofloxacin resistance of the efflux pump inhibitor for multiple resistance strains

After performing disc susceptibility testing, 14 strains from each sample origin: Extensive indoor production system, organic production system, intensive production system and human stool, and 24 strains from retail poultry products origin were selected according to resistance profiles previously determined. For the present work we considered that multiple resistances occur when resistance is found for more than 2 antibiotic classes (ESCMID, 2011). Minimum inhibitory concentrations, MICs, were performed on each of the selected strains, for erythromycin, ciprofloxacin, tetracycline and gentamicin as well as for BC and SH.

The putative contribution of an efflux pump on intrinsic and high levels of resistance was under evaluation at this point. The main efflux pump involved in fluoroquinolone resistance in *Campylobacter* has been characterized as a member of the RND superfamily (Fàbrega *et al*, 2008). By adding an efflux-pump inhibitor (PAβN) one would conclude upon the contribution of an efflux pump, potentially the CmeABC, on antimicrobial resistance, specifically on ciprofloxacin resistance. In the revised literature, Payot *et al.* (2004); Cagliero *et al.* (2006); Hannula & Hänninen, (2008), among others there was a detailed description for macrolide resistance, namely erythromycin, and the effect of putative efflux pumps on the resistance patterns in *Campylobacter* spp.. For ciprofloxacin however, the amount of available information was somewhat restricted.

3.7.1. Characterization of selected strains for antibiotic multiple resistance regarding genes and phenotypic expression

Extensive indoor production system selected strains

From the 14 selected strains of extensive indoor production system origin, 8 were *C. jejuni* (57%) and 6 were *C. coli* (43%). After MIC testing was performed for both antibiotics and disinfectants it was observed that the 14 selected strains were actually completely susceptible to gentamicin, erythromycin and tetracycline (**Table 17**). Twelve of the 14 tested strains were ciprofloxacin resistant strains and the point mutation responsible for alteration of gyrA was found for all phenotypically ciprofloxacin resistant strains. Although 2 of these strains were identified has having the ability of growing on higher levels of ciprofloxacin concentration (till 32 mg/L) non were considered HLR. These same two strains were also resistant to SH growing till 128mg/L concentration steps. Resistance to BC was found in 4 strains of which 2 coincided with SH and ciprofloxacin resistance. One strain was simultaneously resistant to higher concentration levels ciprofloxacin (32 mg/L), BC (4 mg/L) and SH (128 mg/L).

Organic production system selected strains

From the 14 selected strains of organic production system origin, 3 were *C. jejuni* (n=3, 21%) and 11 were *C. coli* (n=11, 79%). After performing MICs for both antibiotics and disinfectants it was observed that for both tetracycline and gentamicin susceptibility was found for all tested strains (n=14 of N=14) (**Table 18**). The exact reverse situation was found for erythromycin for which all strains were evaluated as resistant. For the fluoroquinolone ciprofloxacin, 4 strains were practically entirely susceptible to the antibiotic, 6 were borderline susceptible considering that growth stopped at 0.5 mg/L, and 4 strains were found to be resistant, these resistant strains stopped growing between 8mg/L and 512mg/L. All of these strains carried the *gyrA* point mutation but only 3 were HLR. As far as the disinfectants are concerned only one strain was resistant to both. This one strain though, strain B1, showed resistance levels as high as 4mg/L for the quaternary ammonium BC and even higher for SH, 512 mg/L. B1 also persisted in the presence of 512 mg/L of ciprofloxacin and 128 mg/L for erythromycin.

Table 17 - Comprehensive resume for extensive production strains (EI), regarding gene and biocide resistance phenotypic expression. C.c, *C. coli*; C.j, *C. jejuni*; G, gentamicin; TE, tetracycline; E, erythromycin; CIP, ciprofloxacin; BC, benzalkonium chloride; SH, sodium hypochloride. ERY2074/2075 point mutation for erythromycin resistance; gyrA^{MUT} for ciprofloxacin resistance. Red stands for resistant, green for susceptible; -/- refers to not detected/not detected; -/+, not detected/ detected.

	Strains													
	EI1	EI2	EI3	EI4	EI5	EI6	EI7	EI8	EI9	EI10	EI11	EI12	EI13	EI14
Specie	*C.c*	*C.c*	*C.j*	*C.j*	*C.c*	*C.c*	*C.c*	*C.j*	*C.j*	*C.j*	*C.c*	*C.j*	*C.j*	*C.j*
G	green	green	green	green	green	green	green	green	green	green	green	green	green	green
TE	green	green	green	green	green	green	green	green	green	green	green	green	green	green
E	green	green	green	green	green	green	green	green	green	green	green	green	green	green
CIP	green	red	green	red	red	red	red	red	red	red	red	red	red	red
BC	green	green	green	red	green	green	red	green	green	green	green	green	red	red
SH	green	green	green	green	green	green	red	green	green	green	green	red	green	red
ERY2074/2075	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
gyrA ^{MUT}	-	+	-	+	+	+	+	+	+	+	+	+	+	+

Table 18 - Comprehensive resume for organic production strains (O), regarding gene and antimicrobial resistance phenotypic expression. C.c, *C. coli*; C.j, *C. jejuni*; G, gentamicin; TE, tetracycline; E, erythromycin; CIP, ciprofloxacin; BC, benzalkonium chloride; SH, sodium hypochloride. ERY2074/2075 point mutation for erythromycin resistance; gyrAMUT for ciprofloxacin resistance. Red stands for resistant, green for susceptible; -/- refers to not detected/not detected; -/+, not detected/ detected and HLR, high level resistance.

	Strains													
	O1	O2	O3	O4	O5	O6	O7	O8	O9	O10	O11	O12	O13	O14
Species	*C.c*	*C.j*	*C.c*	*C.c*	*C.c*	*C.c*	*C.c*	*C.c*	*C.c*	*C.c*	*C.c*	*C.j*	*C.j*	*C.c*
G	green	green	green	green	green	green	green	green	green	green	green	green	green	green
TE	green	green	green	green	green	green	green	green	green	green	green	green	green	green
E	HLR (red)	red	red	red	red	red	red	red	red	red	red	red	red	red
CIP	HLR (red)	green	green	HLR (red)	red	green	HLR (red)	green	green	green	green	green	green	green
BC	red	green	green	green	green	green	green	green	green	green	green	green	green	green
SH	red	green	green	green	green	green	green	green	green	green	green	green	green	green
ERY2074/2075	-/+	-/+	-/-	-/+	-/+	-/-	-/-	-/-	-/-	-/-	-/-	-/+	-/-	-/+
gyrA ^{MUT}	+	+	-	+	+	-	+	-	-	-	+	-	-	-

Intensive production system selected strains

From the 14 selected strains of intensive production system origin, 6 were *C. jejuni* (n=6, 43%) and 8 were *C. coli* (n=8, 57%). All strains were susceptible to gentamicin and resistant to ciprofloxacin, till 16mg/L, although none were classified as HLR. Five strains were resistant to tetracycline growing even at antibiotic concentrations of 64mg/L and 3 strains, all HLR, were erythromycin resistant (**Table 19**). The same 2 strains were resistant to both disinfectants growing till 64mg/L for SH and till 4mg/L for BC. Strain I14 was resistant to all antimicrobials tested except for gentamicin.

Table 19 - Comprehensive resume for intensive production strains (I), regarding gene and antimicrobial resistance phenotypic expression. C.c, *C. coli*; C.j, *C. jejuni;* G, gentamicin; TE, tetracycline; E, erythromycin; CIP, ciprofloxacin; BC, benzalkonium chloride; SH, sodium hypochloride. ERY2074/2075 point mutation for erythromycin resistance; gyrAMUT for ciprofloxacin resistance. Red stands for resistant, green for susceptible; -/- refers to not detected/not detected; -/+, not detected/ detected; HLR, high level resistance.

	Strains													
	I1	I2	I3	I4	I5	I6	I7	I8	I9	I10	I11	I12	I13	I14
Specie	*C.c*	*C.c*	*C.j*	*C.j*	*C.j*	*C.c*	*C.c*	*C.c*	*C.j*	*C.j*	*C.j*	*C.c*	*C.c*	*C.c*
G	[green]	[green]	[green]	[green]	[green]	[green]	[green]	[green]	[green]	[green]	[green]	[green]	[green]	[green]
TE	[green]	[green]	[green]	[green]	[green]	[green]	[green]	[green]	[green]	[red]	[red]	[red]	[red]	[red]
E	[green]	[green]	[green]	[green]	[green]	[green]	[green]	[green]	[green]	[green]	**HLR** [red]	**HLR** [red]	[green]	**HLR** [red]
CIP	[red]	[red]	[red]	[red]	[red]	[red]	[red]	[red]	[red]	[red]	[red]	[red]	[red]	[red]
BC	[green]	[green]	[green]	[green]	[green]	[red]	[green]	[green]	[green]	[green]	[green]	[green]	[green]	[red]
SH	[green]	[green]	[green]	[green]	[green]	[red]	[green]	[green]	[green]	[green]	[green]	[green]	[green]	[red]
ERY2074/2075	-/-	-/-	-/+	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/+	-/+	-/-	-/+
gyrA ^{MUT}	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Human stool samples selected strains

From the 14 selected strains of human stool origin, 8 were *C. jejuni* (57%) and 6 were *C. coli* (43%). Once again all strains were susceptible to gentamicin (**Table 20**). Four of the 5 strains resistant to tetracycline grew till antibiotic concentration reached 64mg/L. Of the 14 strains tested 2 were shyly resistant to erythromycin while all strains were resistant to ciprofloxacin. Only one strain was resistant to the effect of the disinfectants, the same strain for both BC and SH. This strain was also resistant to ciprofloxacin.

Retail selected samples

All the 24 selected strains of retail origin were *C. coli* identified strains. Amongst them was no gentamicin resistant strain. 7 out of the 24 tested strains were resistant to tetracycline and 11 showed resistance levels to erythromycin (**tables 21 and 22**). Only 3 strains of the selected 24 were susceptible to ciprofloxacin while only 3 were susceptible to the disinfectant BC. All strains were resistant do SH.

Table 20 - Comprehensive resume for human strains (H), regarding gene and antimicrobial resistance phenotypic expression. C.c, *C. coli*; C.j, *C. jejuni;* G, gentamicin; TE, tetracycline; E, erythromycin; CIP, ciprofloxacin; BC, benzalkonium chloride; SH, sodium hypochloride. ERY2074/2075 point mutation for erythromycin resistance; gyrAMUT for ciprofloxacin resistance. Red stands for resistant, green for susceptible; -/- refers to not detected/not detected; -/+, not detected/ detected.

	Strains													
	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13	H14
Specie	*C.j*	*C.j*	*C.j*	*C.j*	*C.j*	*C.c*	*C.c*	*C.c*	*C.c*	*C.c*	*C.c*	*C.j*	*C.j*	*C.j*
G	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green
TE	Red	Green	Green	Red	Green	Green	Green	Green	Red	Green	Green	Red	Red	Green
E	Green	Green	Green	Green	Green	Green	Green	Green	Red	Green	Green	Red	Green	Green
CIP	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red
BC	Green	Green	Green	Green	Green	Green	Green	Green	Green	Red	Green	Green	Green	Green
SH	Green	Green	Green	Green	Green	Green	Green	Green	Green	Red	Green	Green	Green	Green
ERY2074/2075	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/+	-/-	-/-	-/+	-/-	-/-
gyrA ^{MUT}	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 21 - Comprehensive resume for processed poultry retail strains regarding gene and antimicrobial resistance phenotypic expression. C.c, *C. coli*; C.j, *C. jejuni;* G, gentamicin; TE, tetracycline; E, erythromycin; CIP, ciprofloxacin; BC, benzalkonium chloride; SH, sodium hypochloride. ERY2074/2075 point mutation for erythromycin resistance; gyrAMUT for ciprofloxacin resistance. Red stands for resistant, green for susceptible; -/- refers to not detected/not detected; -/+, not detected/ detected.

	Strains											
	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	R12
Specie	*C.c*	*C.c*	*C.c*	*C.c*	*C.c*	*C.c*	*C.c*	*C.c*	*C.c*	*C.c*	*C.c*	*C.c*
G	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green
TE	Red	Green	Red	Red	Red	Red	Green	Red	Green	Green	Green	Green
E	Red	Green	Green	Green	Green	Green	Red	Green	Green	Red	Red	Green
CIP	Red	Green	Green	Red	Red	Red	Red	Red	Red	Red	Red	Green
BC	Green	Green	Green	Green	Green	Red	Red	Red	Green	Green	Green	Green
SH	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red
ERY2074/2075	-/+	-/-	-/-	-/-	-/-	-/-	-/+	-/-	-/-	-/+	-/+	-/-
gyrA ^{MUT}	+	-	-	+	+	+	+	+	+	+	+	-

Table 22 – Continuation of the comprehensive resume for processed poultry retail strains regarding gene and antimicrobial resistance phenotypic expression. C.c, *C. coli*; C.j, *C. jejuni;* G, gentamicin; TE, tetracycline; E, erythromycin; CIP, ciprofloxacin; BC, benzalkonium chloride; SH, sodium hypochloride. ERY2074/2075 point mutation for erythromycin resistance; gyrAMUT for ciprofloxacin resistance. Red stands for resistant, green for susceptible; -/- refers to not detected/not detected; -/+, not detected/ detected; HLR, high level resistance.

	Strains											
	R13	R14	R15	R16	R17	R18	R19	R20	R21	R22	R23	R24
Specie	*C.c*	*C.c*	*C.c*	*C.c*	*C.c*	*C.c*	*C.c*	*C.c*	*C.c*	*C.c*	*C.c*	*C.c*
G	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green
TE	Green	Green	Green	Green	Green	Green	Green	Red	Green	Green	Green	Green
E	Green	Green	Red	Green	Red	Red	Green	Red	Green	Red	Red	Red
CIP	Red	Red	Red	Red	Red	Red	Red	Red **HLR**	Red	Red	Red	Red
BC	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green
SH	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red
ERY2074/2075	-/-	-/-	-/+	-/-	-/+	-/+	-/-	-/+	-/-	-/+	-/+	-/+
gyrA ^{MUT}	+	+	+	+	+	+	+	+	+	+	+	+

Special consideration was taken for the results concerning erythromycin and ciprofloxacin resistance primarily due to the fact that both these antibiotics are used as therapeutic agents when human *Campylobacter* infection so requires. According to Mammelli *et al.* (2005), Payot *et al.* (2004) and Kurinčič *et al.* (2007) *Campylobacter* isolates exhibit two different phenotypes regarding erythromycin and ciprofloxacin resistance: High-level resistant (HLR) and low-level resistant (LLR). According to Mammelli *et al.* (2005), LLR designation is attributed to strains with growth levels for borderline breakpoints values as defined by *CA-SFM* (2009), therefore LLR erythromycin strains would stop growing at 4mg/L and LLR ciprofloxacin strains stop growing at 1 mg/L. HLR was attributed to strains that persisted till and beyond 64 mg/L for both the antibiotics mentioned. Considering the total 80 selected strains from different origins, 83% (n=66) were of poultry origin and 18% (n=14) were of human origin. Erythromycin HLR was attributed to 4 strains, 1 of organic system origin and 3 of intensive production system. Erythromycin LLR resistance was not attributed to any of the tested strains. Ciprofloxacin HLR was identified in 4 of the total 80 selected strains: 3 of organic production system and 1 of retail sample origin. No HLR strains for either antibiotic were detected for human strains. Once more no LLR ciprofloxacin resistant strains were identified among the total selected strains.

As described by Payot *et al.* (2004), while none carried the A2074G, all the erythromycin HLR strains carried the point mutation A2075G, detected by MAMA-PCR. The latter is one of the most predominant mutations among the erythromycin-resistant strains. We found, however, in the present study, that the occurrence of such point mutation is not exclusive of HLR strains given that the mutation was also detected for erythromycin resistant, but not HLR, strains. Phenotypically identified resistant isolates that did not carry neither point mutations, 2074 nor 2075, were also identified. The occurrence of these resistant phenotypes may be attributed to the occurrence other, alternative, resistance mechanism, as described by Gibreel & Taylor (2006). A high level of antimicrobial resistance, especially for sodium hypochloride, was generally observed among strains (n=31) and more so for processed poultry meat retail products, for which all strains were HS resistant. This may be due to the fact that this disinfectant is widely used along the meat processing chain. Strains frequently come in contact with this compound and in time developed resistance to it (Sprenger, 1993; Moore *et al*., 2006). Tested strains showed resistance levels that ranged from 0 to 512mg/L. 11 of the tested strains were benzalconium chloride resistant. All strains resistant to the quaternary ammonium compound were also resistant to sodium hypochloride.

It is also worth mentioning the occurrence of a quite evident parallelism between ciprofloxacin resistance and biocide resistance. Cross-resistance though is yet to be corroborated leaving the question of antibiotic resistant by disinfectant molecules still open for evidences.

3.7.2.Efflux pump inhibition effect on ciprofloxacin resistant strains

The existence of an efflux pump responsible for the extrusion of different compounds, including antibiotics and disinfectants is now a generally accepted fact. The pump is constitutively expressed in wild type *Campylobacter* contributing to intrinsic resistance. The efflux pump inhibitor, phenylalanine arginine β-naphthylamide (PAβN) has been described as an efficient EPI, restoring erythromycin- and ciprofloxacin- susceptibility to resistant *Campylobacter* strains (Martinez & Lin, 2006). The effect of the efflux pump on ciprofloxacin resistance was tested in the present study. Ciprofloxacin resistance has been attributed, by several authors: Charvalos *et al.*, (1995); Häninnen & Hannula, (2007), to the occurrence of point mutations in the *gyrA* gene, the molecular target of these agents. Such mutations include specific point mutations leading to substitutions at Ala-70, Asp-90 and Thr-86, being the latter the most frequently detected. Point mutations are generally regarded as responsible for HLR profiles while LLR or intrinsic resistance is associated with the presence of efflux pumps that actively extrude biocidal compounds (Mamelli *et al.*, 2005).

Intensive production system selected strains

For all 10 ciprofloxacin resistant strains even the presence of a low PAβN dose of 10mg/L was sufficient to entirely restore ciprofloxacin susceptibility (**Figure 14**). Two of these strains were able to grow at a ciprofloxacin concentration of 32 mg/L even for more one-fold of EPI inhibitor concentration maintaining the resistant phenotype. At 40mg/L of inhibitor concentration susceptibility was restored. A similar outcome was reported by Payot *et al.* (2004) for which optimal inhibition was obtained with 40 mg/l of EPI.

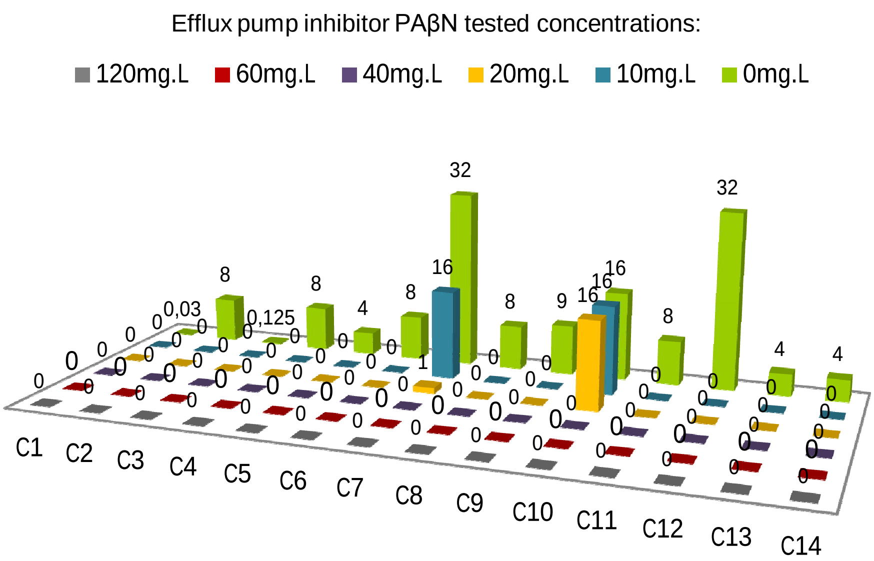

Figure 14 – Efflux pump inhibitor tested concentrations (0mg/L; 10mg/L; 20mg/L; 40mg/L; 60mg/L and 120mg/L) for the 14 selected extensive indoor strains at ciprofloxacin concentrations raging from 0.03mg/L till 512mg/L.

Organic production system selected strains

The same pattern was observed for organic production system selected strains for which 3 strains were identified as ciprofloxacin HLR. However, for this HLR strains susceptibility was not restored even at PAβN concentrations as high as 120mg/L (**Figure 15**). This was not an expected outcome for to our knowledge 40 mg/L, as described by Payot *et al.* (2004) was the optimal EPI, and by all means the maximum needed concentration for restoring ciprofloxacin susceptibility. To our knowledge there are no current reports covering this situation. Still, a 10 mg/L concentration for the inhibitor was enough to restore susceptibility to all resistant strains.

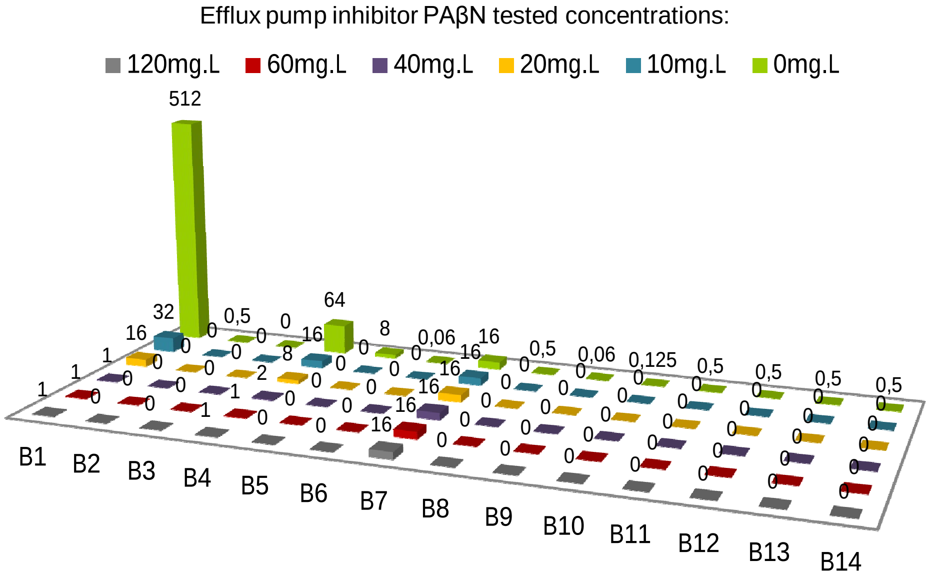

Figure 15 - Efflux pump inhibitor tested concentrations (0mg/L; 10mg/L; 20mg/L; 40mg/L; 60mg/L and 120mg/L) for the 14 selected organic strains at ciprofloxacin concentrations raging from 0.03mg/L till 512mg/L.

Intensive production system selected strains

Intensive production system selected strains had similar responses to those observed for organic production system strains except for the fact that, two strains, not classified as HLR still maintained resistance to ciprofloxacin till EPI concentrations of 60mg/L (**Figure 16**). only becoming slightly more sensitive to the antibiotics effect at the next fold concentration of 120 mg/L at which one strain, still growing, became ciprofloxacin susceptible while the other maintain the resistant status. All resistant strains carried the point mutation responsible for Thr-86- Ile exchange.

Human selected stool sample origin strains

All human stool samples origin strains, were ciprofloxacin resistant and all carried the previously mentioned codon-exchange responsible point mutation in QRDR region. 2 high resistance, but not HLR strains, grew without subsiding, till inhibitor concentrations of 120 mg/L. Four other resistant strains grew in the presence of 10mg/L but such growth was completely abolish in the next one-fold step of 20 mg/L EPI concentration (**Figure 17**).

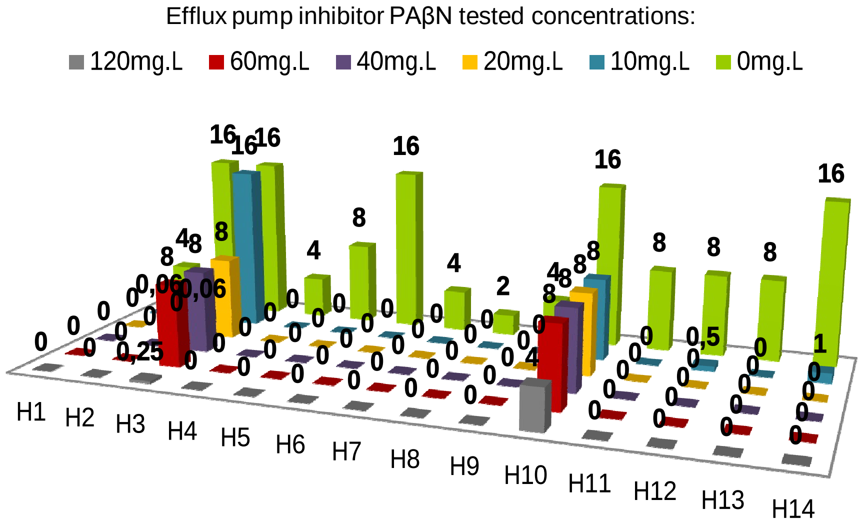

Figure 16 - Efflux pump inhibitor tested concentrations (0mg/L; 10mg/L; 20mg/L; 40mg/L; 60mg/L and 120mg/L) for the 14 selected intensive strains at ciprofloxacin concentrations raging from 0.03mg/L till 512mg/L.

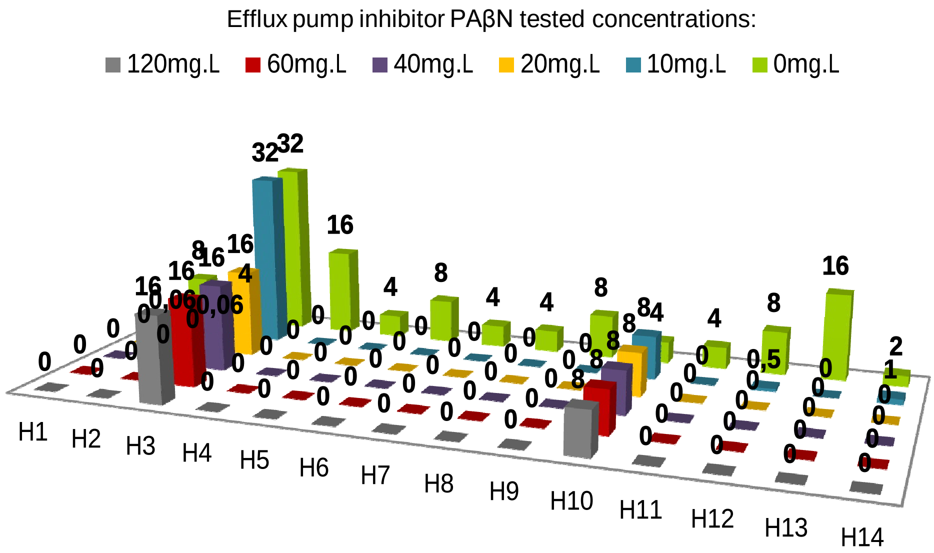

Figure 17 - Efflux pump inhibitor tested concentrations (0mg/L; 10mg/L; 20mg/L; 40mg/L; 60mg/L and 120mg/L) for the 14 selected human strains at ciprofloxacin concentrations raging from 0.03mg/L till 512mg/L.

Retail selected samples

Finally for the 24 retail selected samples one could observe that in the presence of the lowest tested EPI concentration of 10 mg/L strains stopped growing (**Figure 18**). By an EPI concentration of 20 mg/L only higher and HLR strains still grew in the presence of 120 mg/L PAβN concentrations.

Our results indicate that, even for those strains carrying the point mutation, Thr-86- Ile, to which higher levels of resistant are attributed, ciprofloxacin susceptibility was, in general, restored. Some strains that revealed higher but not HLR, also seem to resist high levels EPI. The growth persistence of these non ciprofloxacin HLR strains, even at higher inhibitor concentrations may be attributed to the fact that these strains, although not phenotypically classified as HLR carried other mutations besides the point mutation in the QRDR of *gyrA* responsible for the Thr-86-Ile modification or possessed alternative active efflux systems since many are only now being discovered and/or characterized (Hannula & Hänninen, 2008).

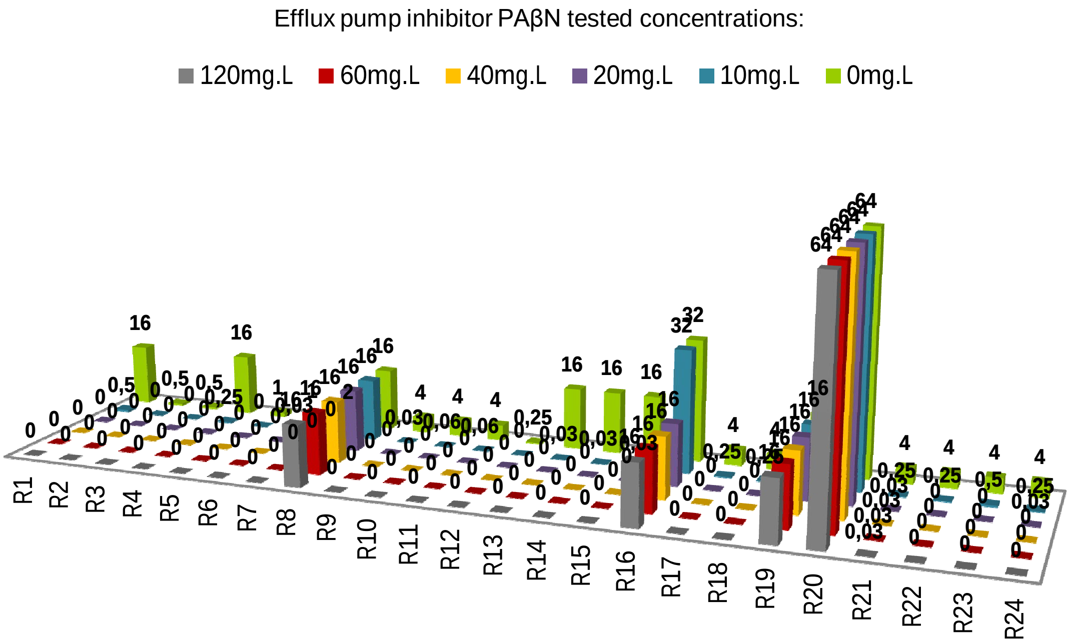

Figure 18 - Efflux pump inhibitor tested concentrations (0mg/L; 10mg/L; 20mg/L; 40mg/L; 60mg/L and 120mg/L) for the 14 selected retail strains at ciprofloxacin concentrations raging from 0.03mg/L till 512mg/L.

4. Conclusions

As the incidence of Campylobacteriosis continues to rise so rises the amount of multiple resistant strains. This alarming situation constitutes a serious health burden for which there is a pressing need for the development of new and better report and control protocols harmonized throughout the European Union, and Portugal is no exception.

The purpose of this work was the phenotypical and genotypical characterization of isolates from different origins: extensive indoor, organic and intensive production systems, human stool samples from patients with Campylobacteriosis and retail processed poultry products in order the perceive the general panorama regarding antimicrobial resistance in both poultry and human strains in Portugal.

From the total initial number of poultry, human and retail origin isolates, 43% were identified as *Campylobacter jejuni* while 57% were *Campylobacter coli* strains. Three fourths, 75%, of tested isolates were of poultry meat origin while the remaining isolates were of human *Campylobacter*iosis patients' origin.

For the total poultry, human and retail origin strains, was observed a high resistance frequency to fluoroquinolones antibiotic tested (between 58% and 92%). For erythromycin, 30% to 40% of studied isolates were resistant. Isolates revealed resistance levels of 6% to 45% for gentamicin. Resistance pattern for tetracycline ranged from 37% to 91%.

Poultry strains of extensive indoor production system isolates had the lowest ciprofloxacin resistance level when compared to organic production systems. Retail product strains had the highest level of all ciprofloxacin resistance isolates; this may be due to the occurrence of cross-resistance between disinfectant and preservative molecules to which meat products are subjected. Observations made for fluoroquinolones were similar to those of tetracycline, with extensive indoor production system strains having the lowest resistance patterns when compared to both organic and retail samples. Once again retail samples had the highest level of resistance observed, 91% among meat poultry samples. For extensive indoor production samples erythromycin resistance was observed for 75% of isolates while 40% was found for retail isolates.

Human isolates showed high level percentage of resistant isolates to all therapeutic alternatives. 37% of isolates were tetracycline resistant and more than 70% were resistant to ciprofloxacin. Although a small fraction of gentamicin resistant isolates were attained from human samples, making gentamicin an attractive alternative to antimicrobial therapy in humans, this antibiotic is highly toxic to humans, being both nephro and hepatotoxic, and thus its utilization is highly restricted.

Multiple drug resistance was one of the major issues to be considered in the present work. We observed that for all tested isolates, most strains were settled at resistance to 4 or more antibiotic classes (quinolones, macrolides, tetracycline and β-lactamics) for each different origin. Multiple resistances occurred from 86% till 93% of tested strains. Human and retail strains had no completely susceptible strains.

All the strains considered HLR for erythromycin and ciprofloxacin carried the respectively point mutations: A2075G and gyrA (Thr86Ile). However other resistance strains that were not HLR also carried the same mutations. The maximum MIC concentration attained for erythromycin was 128 mg/L and for ciprofloxacin was 512 mg/L and still growing. Both results pertained to an organic strain, O1. On average, the majority of strains were ciprofloxacin resistant between 8 mg/L and 16 mg/L.

For biocide resistance tested, a parallelism was observed between some HLR ciprofloxacin strains and BC and SH resistance patterns. The maximum resistance concentration registered was 64 mg/L for BC and 512 mg/L of SH. Retail strains showed the highest levels, 100%, of resistance to the sodium hypochloride biocide.

Considering the effect of the efflux pump inhibitor tested (PAβN) to ciprofloxacin resistance, the HLR strains persisted even when confronted with high level concentrations of 120mg/L. For the remaining strains susceptibility to ciprofloxacin was entirely restored between 10 mg/L and 40 mg/L.

In summary, the worldwide combination of data along with conventional and molecular epidemiological information, will allow for a better understanding of how antibiotic resistance is initiated, acquired and how such organisms may be transmitted to new animal and human hosts. Currently, the general inadequate use of antibiotics, such as quinolones and macrolides is a matter of grave concern due to its contribution to the acquisition of resistance in foodborne bacteria that potentially leads to limitations in the efficacy of such compounds in treating human infections. There appears to be a general tendency for antibiotic resistance rates to rise against several antibiotic agents and thus multiple resistance patterns to several classes of antibiotics are beginning to emerge and Portugal seems to be no exception.

Future work should include the efflux pump inhibitor potential in overcoming antibiotic resistance, in prevention and control of *Campylobacter* infection in humans and animal reservoirs with a potential use as an alternative drug therapy. The effect of the efflux pump inhibitors on biocide resistance strains should be performed. Establishment of evidences corroborating the occurrence of cross-resistance between antibiotics and biocides is also in need of clarification, since the future interventions for *Campylobacter* control underlines the use of biocides on carcass decontamination besides their commun use on hygienization plans for equipments and premises on food Industry. New and more complete information is also needed regarding resistance patterns and mechanisms of protection associated with stress responses, such as biofilm development.

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