University of Naples Federico II

Doctorate in Food Science

XXXIII Cycle

Isolation and characterization of *Campylobacter* spp. in meat products

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Academic Year 2019-20

Alle mie figlie Vicky e Azzurra
e al mio adorato
Matteo

"....l'importante nella
vita non è ciò che farete
ma come lo farete...."

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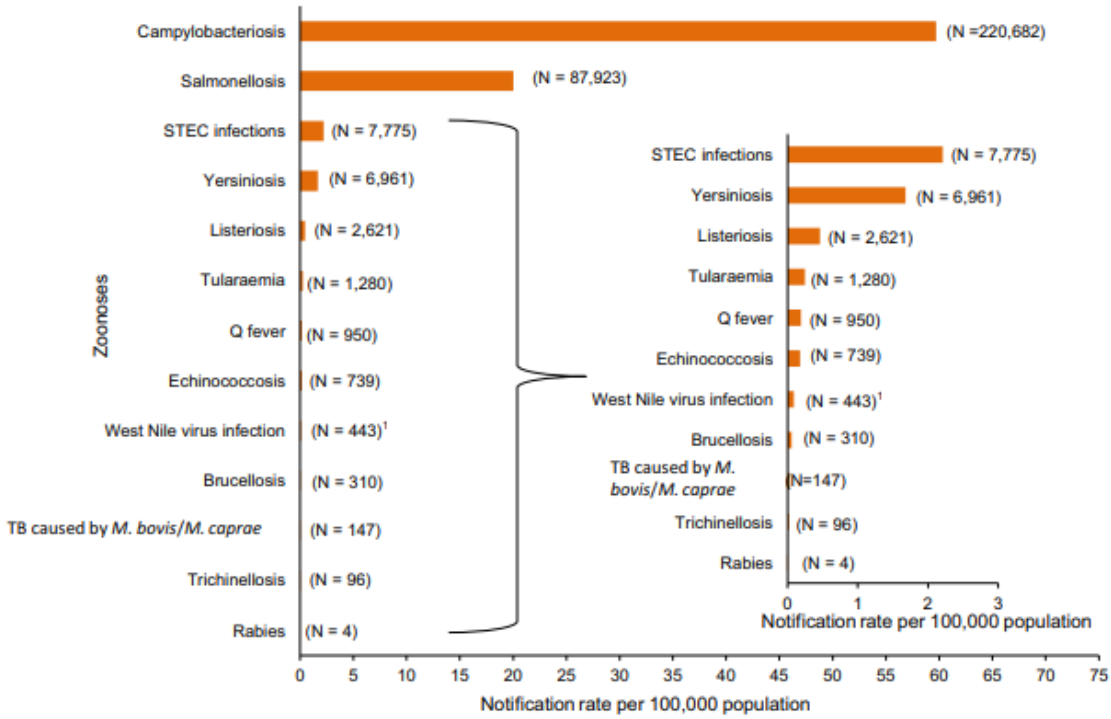
INTRODUCTION

1. State of the art

Campylobacter is an emerging pathogen that has aroused concern in recent years. This Gram-negative microorganism causes gastroenteritis, which may, in some cases, lead to severe complications. Only a few years ago, Campylobacteriosis, as this disease is called, was indicated as a rare bacteremia in immunocompromised subjects. In 1972, *Campylobacter* infection was acknowledged to be responsible for diarrheal diseases, and, for the last 20 years, it has been recognized as the cause of dangerous pathologies, such as Guillain-Barré syndrome. The European Food Safety Authority (EFSA), in its annual report on infectious diseases that are transmissible from animals to humans, publishes data on the incidence of the main infectious diseases in the European Union; the latest reports have indicated that *Campylobacter* is the leading cause of zoonosis in Europe (Figure 1).



Note: The total number of confirmed cases is indicated between parentheses at the end of each bar.
[1] Exception: West Nile virus infection for which the total number of cases was used.

Figure 1: Reported numbers and notification rates of confirmed human zoonoses in the EU, 2019

Campylobacteriosis is one of the most widespread gastrointestinal bacterial diseases in the world, and in some European countries its incidence rate has overtaken that of non-typhoidal salmonelloses. Most infections are caused by the species *C. jejuni* and *C. coli*, while those caused by *C. lari*, *C. fetus* and *C. upsaliensis* are less frequent. In 2019I information on the *Campylobacter* species involved was provided by 24 EU member states in 55.2% of the confirmed cases

reported in the EU, a percentage similar to the 2018 value. Of these, 83.1% were *Campylobacter jejuni*, 10.8% *Campylobacter coli*, 0.1% *Campylobacter lari*, 0.1% *Campylobacter fetus* and 0.1% *Campylobacter upsaliensis*, as reported by The European Union On eHealth 2019 Zoonoses Report. In 2019, cases of infection in Europe totaled 220,682. The main source of the most common infections by Campylobacter spp was poultry; of 3746 skin samples from refrigerated carcasses of broiler hens, 34.6% tested positive (Scientific Report, EFSA 2019).

The same EFSA report also provided data on the antibiotic resistance of *Campylobacter* spp from farm animals and foodstuffs of animal origin. The phenomenon of antibiotic resistance has now become a worldwide concern and is steadily growing. Indeed, the percentages of bacteria that are resistant to the antibiotics usually used to treat human infections are increasing; this means that treatment with the most common antibiotics often proves inefficacious.

Campylobacter normally colonizes the intestine of many animals, both wild and domestic, and during the various phases of butchery, meats may become contaminated. In this regard, poultry meat has been seen to be the most frequently and heavily contaminated. *Campylobacter* may also be found in unpasteurized cow's milk, and studies have shown that this microorganism may be present in untreated sewage and surface waters (Lapo Mughini et al., 2016). In the past, contaminated water was regarded as a major source of *Campylobacter* spp. Indeed, *Campylobacter* spp. are commonly found in surface waters of farms (Mughini-Gras et al., 2016), and water that has not been adequately treated can act as a vehicle of

transmission (Jonsson et al., 2012; Agunos et al., 2014; Allain et al., 2014; Torralbo et al., 2014; Borck Høg et al., 2016).

Outbreaks of *Campylobacter* infections are sometimes difficult to identify (Teunis et al., 2013). However, where outbreaks have been thoroughly investigated, it has emerged that the most common vehicles of infection are undercooked meat, especially poultry, and unpasteurized milk used in cheese production or consumed during organized visits to farms. Other potential sources of infection are water that is untreated or stored in contaminated containers, and inadequately cooked pork or lamb (Table 1).

Table 1.Occurrence of *Campylobacter* in major food categories, EU

Food	2019			2015–2018		
	N reporting MS	N sampling units	Positive N (%)	N reporting MS	N sampling units	Positive N (%)
RTE food						
All	8	3,691	6 (0.16)	15	7,272	36 (0.50)
Meat and meat products	6	328	0	9	1,040	27 (2.60)
Meat and meat products from broilers	1	18	0	3	117	22 (18.80)
Milk and milk products	6	821	2 (0.24)	11	2,258	8 (0.35)
Milk	5	204	2 (0.98)	6	675	6 (0.89)
Raw milk[a]	4	185	2 (1.08)	5	652	6 (0.92)
Cheese	4	615	0	7	1,566	2 (0.13)
Dairy products excluding cheeses (butter, cream, ice cream, whey, yoghurt and fermented dairy products)	2	3	0	4	71	0
Fruits, vegetables and juices	2	1,008	2 (0.20)	4	1,119	1 (0.09)
Salads	5	309	1 (0.32)	2	30	0
Other processed food products and prepared dishes	4	1,002	1 (0.1)	7	2,564	0
Non-RTE food						
All	16	26,687	5,504 (20.62)	20	54,295	13,892 (25.59)
Meat and meat products	15	23,837	5,475 (22.97)	20	49,959	13,817 (27.66)
Fresh meat from broilers	12	8,325	2,464 (29.60)	19	31,665	12,210 (38.56)
Fresh meat from turkeys	6	336	111 (33.04)	8	3,384	824 (24.35)
Fresh meat from pigs	3	135	6 (4.44)	9	3,459	503 (14.54)
Fresh meat from bovine animals	5	374	7 (1.87)	9	3,959	468 (11.82)
Other fresh meat	8	12,614	2,468 (19.57)	12	4,130	668 (16.17)
Milk and milk products	5	884	18 (2.04)	9	1,552	39 (2.51)
Fruits, vegetables and juices	5	512	1 (0.20)	7	1,803	3 (0.17)
Other food	6	1,454	10 (0.69)	8	981	33 (3.36)

(a): The raw RTE milk sampling units are a subset of the RTE milk.

In 2019, a total of 16 Member States and 4non-Member States reported monitoring data on *Campylobacter* in animals. Most of the samples were from broilers and cattle and all proportions (%) of positive sampling units are shown in Table 2.

Table 2.*Campylobacter* reported in MS and non-MS

	N reporting MS/non-MS	N tested units[a] , EU	Proportion (%) of positive sampling units, EU
Animals			
Broilers	5/2	10,196	13.27
Turkeys	0/1	–	–
Pigs	7/1	1,125	58.58
Bovine animals[b]	6/0	3,493	9.28
Cats and dogs	5/2	1,373	6.85
Other animals[c]	7/3	3,024	12.63

MS: Member State.

(a): The summary statistics were obtained summing all sampling units (single samples, batch samples, animals, slaughter animal batches and herds or flocks).

(b): 'Artificial insemination stations' in 'sampling stage' was not included in the count of the units tested.

(c): Antelopes, badgers, birds, bison, budgerigars, canary, Cantabrian chamois, chinchillas, deer, dolphin, ferrets, foxes, geese, goats, guinea pigs, hamsters, hares, hedgehogs, lion, lynx, marten, minks, monkeys, night herons, oscine birds, other animals, parrots, peafowl, pheasants, pigeons, rabbits, raccoons, ratites (ostrich, emu, nandu), rats, reindeers, reptiles, rodents, sheep, snakes, domestic solipeds, Steinbock, turtles, water buffalos, wild boars, wild ducks, wolves and zoo animals.

Cases of food poisoning very often occur in the home. They are frequently caused by cross-contamination between raw and cooked foods, or by deleterious practices, such as washing chicken under running water before cooking it, thereby spreading any bacteria present to kitchen surfaces. The most frequently identified vehicle of contagion is undoubtedly poultry meat, especially if it is not properly cooked. Foodstuffs of animal origin play a major role in the transmission of *Campylobacter* to humans, asdemonstrated by the fact that the serotypes most frequently isolated from poultry and cattle are also those most frequently isolated from humans (Llarena et al., 2017).

Overall, for the year 2019, 94.5% of the number of reported human campylobacteriosis cases that contracted the infection in the EU (109,930) were domestic infections.

2. Characteristics of the microorganism

The genus *Campylobacter*, from the Greek *kampylos*, which means “curved”, is a non-sporogenic microorganism. However, if situated in an unfavorable environment or in cultures exposed to the air for long periods, it can form coccoid structures. It is a thermophilic, Gram-negative microorganism, but adapts well to temperatures between 30°C and 47°C, displaying optimum growth at 42°C. It has a characteristic curved shape, so much so that when it forms a double "S", it is said to have a "gull-wing" shape; it is oxidase- and catalase-positive, and mobile, owing to its polar flagella. It is sensitive to heat and to desiccation, but resistant to freezing; indeed, it survives better in conditions of refrigeration than at room temperature. Its main antigen is the lipopolysaccharide of the outer membrane. *Campylobacter* grows best in an atmosphere with a low concentration of oxygen (5-7%, microaerophilic) and a high concentration of carbon dioxide (5-10%). The cellular diameter ranges from 0.3 to 0.6 μm; indeed, the cells can pass through filters with 0.45 μm pores, which would trap other bacteria. Table 3 shows the various species of currently known *Campylobacter*.

Table 3.Species of *Campylobacter*, with their main reservoirs/sources and related pathologies

Species	Source	Pathology
C.jejuni ss jejuni	Chickens, pigs, cattle, dogs, cats, birds, minks, rabbits, insects	Gastroenteritis, septicemia, meningitis, abortion, proctitis, Guillan Barrè syndrome
C,jejuni ss doylei	Human	Gastroenteritis, gastritis, septicemia
C.coli	Pigs, chickens, cattle, sheep, birds	Gastroenteritis, septicemia, meningitis, spontaneous abortion
C.lari	Seagull, other birds	Gastroenteritis, septicemia
C.upsaliensis	Dog and cat	Gastroenteritis, emphysema, abscesses
C.hyointestinalis ss hyointestinalis	Pigs, cattle, hamsters, deer	Gastroenteritis
C.hyointestinalis ss lawsonii	Pigs	Gastroenteritis
C.concisus	Human	Periodontal disease
C.rectus	Human	Periodontal disease
C.curvatus	Human	Periodontal disease
C.hyoilei	Human	Variables
C.showae	Human	Periodontal disease
C.fetus ss fetus	Cattle and sheep	Gastroenteritis, miscarriage, septicemia
C.fetus ss veneralis	Cattle	Septicemia
C.mucosalis	Pigs	Hemorrhagic colitis
C.sputorum bv sputorum	Human, cattle, pigs	Gastroenteritis and abscesses
C.sputorum bv fecalis	Sheep, bull	Gastroenteritis
C.sputorum bv paraureolyticus	Mammals	Variables
C.gracilis	Human	Periodontal disease, emphysema and abscesses
C. helveticus	Dog and cat	Variables
C.ureolyticus	Human	Variables
C.canadensis	Birds (grud'America)	Gastroenteritis
C.cuniculorum	Rats	Variables
C. hominis	Human	Gastroenteritis, gastritis, septicemia
C. avium	Poultry	Variables
C. insulanigrae	Marine mammals	Variables

3. Campylobacteriosis

Campylobacter generally causes a gastrointestinal disease called campylobacteriosis. This is not always easy to distinguish from other gastrointestinal diseases, as it does not present specific characteristic clinical manifestations; indeed, abdominal pain, headache and muscle aches are common to many forms of gastroenteritis. The symptoms may be mild or moderate, but the disease may also be acute and self-limiting, characterized by aqueous, and sometimes bloody, diarrhea. The only sign of systemic infection by *Campylobacter* may be fever; this may be constant or intermittent and may oscillate between 38°C and 40°C. Apart from diarrheal disease, *Campylobacter* may sometimes cause sub-acute bacterial endocarditis (generally due to *C. fetus*), meningitis and reactive arthritis, especially of the knee joints.

The incubation period of campylobacteriosis varies from one day to one week, according to the case, and the symptoms may resolve spontaneously in a period ranging from one week to several months. The mortality rate is low, but elderly persons, children and immunocompromised subjects are naturally more susceptible to severe infection. Campylobacteriosis has also been associated with Guillain-Barré syndrome, a polyneuropathy due to demyelination (degeneration of the myelin sheaths that envelop the nerve fibers), which can cause temporary blindness, total paralysis and respiratory insufficiency. Owing to the absence of

specific clinical features, a definitive diagnosis can only be made through the microbiological analysis of clinical samples.

4. Causes and prevention of disease

Campylobacter infections have mainly been associated with the consumption of chicken, of contaminated water or milk, and of raw or lightly cooked at-risk foodstuffs. It therefore follows that the transmission of *Campylobacter* can be curbed by adopting certain precautions: consuming only pasteurized milk; using drinking water only from controlled systems,and banning the use of contaminated or uncontrolled water for the irrigation of crops, especially those that are to be consumed raw. Although the meat of pigs and ruminants is not considered to be at high risk, raw offal is a potential source of dangerous contamination during the process of butchery. Bivalve molluscs that are consumed raw also constitute a major risk.

As mentioned above, the main source of transmission of *Campylobacter* is poultry meat. Contamination may occur during all the phases of preparation, from butchery to consumption: i.e., all the phases of manipulation by both producers and consumers, when the meat may come into contact with fecal matter and/or the contents of the intestines. In this regard, guidelines on risk management should be provided at all levels, in order to raise awareness of the microbiological dangers of the improper handling of products destined for human consumption. Domestic animals may also be reservoirs of *Campylobacter* and facilitate its transmission,

while direct person-to-person contagion is somewhat rare. Washing meat after butchery reduces the risk of contamination, as does freezing. However, the only truly effective ways of eliminating *Campylobacter* from foods are thorough cooking, pasteurization, or irradiation (gamma rays). Although current knowledge of the pathways of contamination of poultry is still incomplete, the factors chiefly correlated with the diffusion of *Campylobacter* are: the level of biosafety, the season, the age of the birds, the feeding modalities used, the conditions of transport, water, and the medicines administered to the animals (Marotta et al, 2015).

5. Pathogenetic factors

The pathogenicity of *Campylobacter* depends on numerous factors, which are related both to the microorganism and to the host. The host's state of health, age and humoral immunity elicited by previous exposure all influence the clinical outcome of infection. The virulence of the microorganism also depends on several factors; the bacterium is endowed with a polar flagellum, which confers motility, thereby facilitating penetration of the mucous barrier of the intestinal epithelium and enabling the bacterium to penetrate and colonize the epithelium. Penetration of this protective mucous barrier is also facilitated by the action of chemotactic tissue factors, the genes responsible for which are *cheA*, *cheB*, *cheR*, and *cheW*. The ability to adhere to and invade the intestinal mucosa depends on the production of certain toxins, and is preliminary to that of mobility; specifically, this involves two

flagellins (*FlaA* and *FlaB*) coded for by the genes *flaA* and *flaB.* When the former gene is suppressed, the mobility of the microorganism is markedly reduced; its ability to penetrate the intestinal mucosatherefore diminishes (Vandamme et al., 2005; Silva et al., 2011). In *Campylobacter jejuni*, motility is also regulated by a sensor, *FlgS*, to which a regulator, *FlgR*, responds; other proteins that are essential for motility (*FlgP* and *FlgQ*) are also involved, while the characteristic corkscrew-like rotary movement that facilitates penetration is regulated by the gene *cheY* (Dasti et al., 2010). Initially, the bacterium is able to attach itself to the epithelial cells owing to the action of a range of adhesins, as well as to that of the flagella themselves. Many genes regulate this adhesive capacity: *cadF, dnaJ, pdlA, racR, capA, virB11, ilvE, peb1A, MOMP/porA.* In the next stage, the bacterium anchors itself to the cell membrane and penetrates the cell by means of a phagocytic-like mechanism. *Campylobacter coli* possesses another two genes, *ciaB* and *pldA*, which confer its invasive capacity (Man, 2011); moreover, other proteins, *CiaB, CiaC, CiaD, Cia1, FlaC, IamA, CeuE, HtrA, VirK*, and *FspA* are responsible for invasion in the various species of *Campylobacter*. Some studies have also shown that the microorganism is able to survive and to exert a toxic effect inside the cells themselves. Enteritis due to *Campylobacter* may be manifested as secretory diarrhea, especially in children in developing countries, or bloody diarrhea. Indeed, the microorganism can act through invasive or toxic mechanisms; in the former case, it causes gastroenteritis, with bloody feces, while in the latter case, it produces toxins: enterotoxins similar to cholera toxin and various cytotoxins. The

production of a *CDT* toxin (Cytolethal Distending Toxin) is considered a factor of virulence. This toxin is made up of three subunits, coded for by three different genes: *cdtA, cdtB* and *cdtC*. The toxin appears to be able to interrupt the cell cycle in the G2/M phase, inhibiting mitosis and causing cell death. The active subunit of the toxin is *cdtB*, while the roles of the subunits *cdtA* and *cdtC* are not yet clearly known. It has nevertheless been hypothesized that these latter are essential to the penetration of the active subunit inside the epithelial cells of the intestinal mucosa. In addition, it has been demonstrated that the CDT toxin is responsible for the production of interleukin IL-8 in humans, with the consequent recruitment of macrophages, dendritic cells and neutrophilic granulocytes to the site of invasion, thereby inducing intestinal inflammation (Dasti et al., 2010). *Campylobacter* also displays a considerable ability to produce biofilm, which enables it to survive inside the host and in the environment. Indeed, these microorganisms can collect in encapsulated aggregates inside an extracellular polymeric substance that can adhere to various surfaces. It is probable that *CosR* is a key protein in the maturation of the biofilm of *C. Jejuni* and that it is also involved in the expression of the *CmeABC* antimicrobial efflux pump (Turonova et al., 2015). Biofilm development may also involve some virulence genes, such as the genes responsible for cell motility *(flaA, flaB, flaC, flaG, fliA, fliS* and *flhA*) and those that act on the cell surface (*peb4, pgp1* and *waaF*), which are responsible for quorum sensing (*luxS*) and the response to stress (*ppk1, spoT, cj1556, csrA, cosR, cprS* and *nuoK*). Furthermore, it must be borne in mind that the surface of the cells of *C. jejuni* is surrounded by a lipo-

oligosaccharide (LOS) made up of a central oligosaccharide and a lipid A, of a capsular polysaccharide (CPS), and of N-linked and O-linked glycosylated proteins. These molecules carry out several functions, including immune functions, adhesion to the host cell and invasion. Finally, a recent interesting discovery is that the strains of *C. jejuni* that do not possess external central parts of the LOS display a markedly increased tendency to form biofilms (Tram et al, 2020; Naitoet al., 2010).

6. Antibiotic Resistance

The phenomenon of antibiotic resistance on the part of several species of *Campylobacter* is increasing; particularly alarming is the resistance to fluoroquinolones. In this regard, some studies have found a correlation between the use of fluoroquinolones on livestock farms and the development of campylobacteriosis caused by fluoroquinolone-resistant strains in both animals and humans.

According to the EFSA, which has reported the data from 2017, in some countries the resistance of bacteria of the genus *Campylobacter* to fluoroquinolones (such as ciprofloxacin) is so great that these antibiotics no longer work in the treatment of severe cases of campylobacteriosis. Although slightly fewer cases were reported in 2017 than in 2016 (246,158 vs 246,917), campylobacteriosis remains the most frequently reported zoonosis in the EU.

In Italy, the latest EFSA report indicates that *Campylobacter coli* displays greater antibiotic resistance than *Campylobacter jejuni*; the percentages are shown in Table 4.

Table 4. Percentages of antibiotic resistance recorded in strains of *C. coli* and *C. jejuni* isolated in Italy in 2020 (source: EFSA Report)

Antibiotic molecles	*C.coli*	*C.jejuni*
TETRACICLYNES	84,0%	58,7%
CIPROFLOXACIN	80,0%	61,8%
ERYTROMYCIN	24,0%	0,0%
CIPROFLOXACIN/ERYTROMYCIN	24,0%	0,0%

The conclusions of the latest report on antibiotic resistance in zoonoses published by the European Centre for Disease Prevention and Control (ECDC) and by the EFSA are as follows. In all EU member states, among the isolates of *C. jejuni* and *C. coli* recovered from poultry meat, the highest levels of resistance were observed against ciprofloxacin, nalidixic acid and tetracycline (overall percentages: 54-83%); resistance was reported to vary from high to extremely high. Ciprofloxacin is one of the fluoroquinolones, a class of antibiotics deemed essential for use in humans. If the fluoroquinolones were to lose their efficacy, the impact on human health would be heavy. The highest levels of resistance were observed in isolates

of*C. coli* recovered from broiler hens (61.4%) and of *C. jejuni* from turkeys (56.1%). Regarding resistance to streptomycin, an antibiotic belonging to the class of aminoglycosides, the highest levels were observed in *C. coli* isolates recovered from fattening pigs (overall, 64.4%), while far lower levels were found in poultry and calves. Overall, moderate levels of resistance to streptomycin were observed in *C. coli* isolated from broiler hens (15.6%) and in *C. jejuni* from calves (15.6%), while low levels were found in *C. jejuni* isolated from broiler hens and fattening turkeys (8.7% and 6.4%, respectively). Resistance to gentamicin in isolates of *C. jejuni* and *C. coli* recovered from poultry meat was not observed in the majority of countries. Resistance to erythromycin was generally higher in *C. coli* isolates (14.3%) than in *C. jejuni* isolates (<2%). With regard to isolates of *Campylobacter* recovered from cecal samples from broiler hens and fattening turkeys, overall resistance to ciprofloxacin and nalidixic acid ranged from high to extremely high; resistance to these antibiotics was generally lower in *C. coli* isolates from fattening pigs (52.3% for both antibiotics) and *C. jejuni* isolates from calves (52.1% for nalidixic acid). Although current knowledge of the pathways of contamination of poultry is still incomplete, the factors chiefly correlated with the diffusion of *Campylobacter* are: the level of biosafety, the season, the age of the birds, the feeding modalities used, the transport of the birds from one farm to another, the conditions of transport, and the water and the medicines administered to the animals. The presence of *Campylobacter* isolates that display combined resistance to both ciprofloxacin and erythromycin is of great concern to public health, as both

compounds are classified as CIA (*Critically Important Antimicrobials*) for the treatment of *Campylobacter* infections in humans (WHO, 2017; EFSA and ECDC,2019). In the treatment of campylobacteriosis, it is essential to rehydrate the patient. Although antibiotic therapy is not usually indicated in cases of moderate enteritis, it may be advantageous in patients at higher risk, such as the elderly, patients with shivering and systemic symptoms, immunocompromised subjects and pregnant women, all of whom present moderate-to-severe dysentery (bloody diarrhea). *Campylobacter* infections can be treated effectively with antibiotics such as erythromycin, tetracycline and fluoroquinolones. However, the widespread use of antibiotics, not only for human therapy, but also in farm animals and even in fish in aquaculture, has led to the selection of pathogenic bacteria that are resistant to several classes of antibiotics, so-called multidrug resistance. With regard to *Campylobacter*, joint resistance to both fluoroquinolones and macrolides is not yet very common. Multidrug resistance can be generated through one of two mechanisms. Firstly, these bacteria may accumulate several genes, each of which codes for resistance to a single drug inside a single cell. This accumulation typically occurs on plasmids of resistance (R). The second factor involved in multidrug resistance is the efflux pump system, a system of self-defense of Gram-negative bacteria, whereby these bacteria utilize quorum sensing in order to regulate the entry of certain harmful substances, including antibiotics, into the cell. Unlike other specific resistances, which enable bacteria to resist only a particular antimicrobial, the acquisition of a functionally potentiated efflux pump will, in the

future, strengthen bacteria with simultaneous resistance to several classes of antibiotics.

OBJECTIVES

The present study is the fruit of a research doctorate carried out in collaboration with the Istituto Zooprofilattico Sperimentale del Mezzogiorno, a body that deals with veterinary public health, food safety and the interplay among the environment, health and human beings. The aim of this research was to study in depth the diffusion and antibiotic resistance of *Campylobcater* spp., a pathogenic microorganism that EFSA sources have identified as the leading cause of zoonoses in Europe in recent years.

EU Regulation 2073/2005 on food safety mandated the official microbiological monitoring of foodstuffs; one specification of this legislation was the need to analyze the carcasses and skin of chickens for the possible presence of *Campylobacter* spp. Nevertheless, regional monitoring plans have been slow to be implemented. The present research focused on chicken and chicken-based preparations, which, though not subject to official investigation, are hypothetically more likely to be contaminated by this pathogen. Moreover, major concern has been aroused by the alarming increase in the resistance of this microorganism to antibiotics, particularly ciprofloxacin and nalidixic acid, and by its marked capacity for gene recombination.

Campylobacter spp. were therefore isolated from at-risk foodstuffs and subjected to preliminary identification by means of molecular and culture techniques. The species considered in the study were *Campylobacter jejuni* and *C. coli* (Chapter 1).

The genomes of the strains isolated were sequenced, and a comparative genomic study that included 1115 genomes of *C. coli* and 2014 genomes of *C. jejuni* present in public databases was undertaken (Chapter 2). Particular attention was focused on the diffusion of antibiotic-resistance genes and on the expression of virulence factors; in this regard, the genomic data were compared with the phenotypic expression of the new isolates (Chapter 3).

CHAPTER 1. Detection, isolation and identification of *Campylobacter* in samples of poultry meat and pork from retail outlets and slaughterhouses.

1. Introduction

The results of the monitoring campaigns coordinated by the EFSA at the European level have revealed the diffusion of *Campylobacter* in foodstuffs, particularly in broiler hens. In 2010, the EFSA published the results of its reference investigation, which was conducted in 2008 in slaughterhouses in order to obtain comparable statistics on the prevalence and level of contamination of meat from chickens in the various EU countries. It emerged that, on average, 75.8% of chicken carcasses were contaminated, with significant variations being observed among member states and slaughterhouses. According to the scientific evidence on the risk of human campylobacteriosis, 20-30% of cases are caused by the handling, preparation and consumption of chicken, while 50-80% of cases can be attributed to the poultry reservoir as a whole. The European regulation that first listed *Campylobacter* among the pathogens to be sought during official inspections of foodstuffs was the European Commission's Reg. (UE) 2017/1495. Issued on 23 August 2017, this modified Reg. (CE) n. 2073/2005 and concerned the carcasses of chickens (Table 1.1). The new regulation established hygiene criteria that set indicative contamination values above which remedial measures were to be implemented in

order to maintain the hygiene of the production process in conformity with legislation on food products.

Table 1.1. European Commission Reg. (UE) 2017/1495 of 23 August 2017

Food category	Microrganism	Sampling plan		Limits		Analytical reference method	Stage where the criterion applies	Action in case of unsatisfactory results
		n	c	m	M			
2.1.9 Carcases of broilers	*Campylobacter* spp.	50(5)	c=20 From1..2020 c=15; From 1.1.2025 c=10	1000 cfu/g		EN ISO 10272-2	Carcassrs after chilling	Improvements in slaughter hygiene, review of process controls, of animals origin and of the biosecurity measures in the farms of origin

Sampling of foodstuffs for the detection and analysis of *Campylobacter* spp is carried out by the Local Health Agencies, which are responsible for official inspections of food products.

2. Materials and Methods

The methods utilized are all validated and accredited according to ISO/IEC 17025 by ACCREDIA, the only national accreditation body designated by the Italian government.

2.1 Microbiology

In the present study, the microbiological analyses carried out in order to isolate *Campylobacter* spp were the official analyses reported in ISO 10272-1:2017 (Horizontal method for detection and numeration of *Campylobacter* spp. – Part 1: Detection method) and ISO TS 10272-2:2017 (Microbiology of the food chain —

Horizontal method for detection and enumeration of *Campylobacter* spp. — Part 2: Colony-count technique). As no official analyses had been carried out, despite being expressly required by the authorities responsible, ISO 10272-1, i.e. qualitative analysis, was used. Twenty-five grams of meat was placed in Bolton enrichment broth (Oxoid) and incubated in microaerophilic conditions at 42°C for 48 hours. The enrichment broth was streaked onto plates of Charcoal Cefoperazone Deoxycholate (CCD) Agar (Oxoid) and incubated at 42°C for 48 hours in microaerophilic conditions. The characteristic colonies were purified on Columbia Agar containing 5% blood.

2.2 Molecular analyses

Molecular analysis for the detection of *Campylobacter* was carried out in accordance with the iQ-Check protocol (IQ-Check *Campylobacter* PCR Detection Kit, Biorad) (http://bio-rad.com/iqcheck), which uses Real-Time PCR technology; it is designed as a multiplex reaction including an internal inhibition control (HEX) that is amplified in parallel with the target DNA (FAM) in order to ensure a reliable negative result.

The PCR iQ-Check *Campylobacter* detection kit is based on a tested, rapid, sensitive technology, whereby the results can be obtained in as little as 24 hours after sample enrichment in a selective Bolton broth.

2.3 Identification

Once the pure characteristic colony had been obtained, species identification was carried out by means of the VITEK®2 (BioMérieux) (Thomas K. W. Ling et al ,2001) system and NH VITEK®2 (BioMérieux) cards. All the VITEK®2 identification cards utilize Advanced Colorimetry™ technology from bioMérieux; this enables species to be distinguished very accurately, while yielding a low rate of multiple choice and species misidentification.

2.4 Results and discussion

A total of 50 samples were analyzed: 46 of chicken and 4 of pork; 29 strains were isolated and presumptively identified as *Campylobacter* spp. Of these, only 25 were able to be cultured after freezing. The subsequent study was therefore carried out on a total of 25 isolates. These were identified as *Campylobacter jejuni* (n=13) and *Campylobacter coli* (n=12). Table 1.2 reports the numbers of strains isolated, broken down by species and source of isolation.

More than 50% of the analyzed samples were found to be contaminated with *Campylobacter* and chicken meat as reported in the literature was the most contaminated. European data show a high prevalence of *Campylobacter* in wild birds and this suggests that the secondary pathways of contamination may have a

significant impact on the actual spread of the pathogen in the food chain, from the environment and the waters.

To date, the most studied species, also because it is more widespread, is *Campylobacter jejuni*, but the number of isolated strains of *Campylobacter coli* is increasing sharply; in fact in this work, the number of *C. coli* and *C.jejuni* isolates is almost the same.

Chicken meat is currently the most contaminated, but the EFSA report shows the incisive increase in positivity in pork. From the samples analyzed in this work, *Campylobacter coli* was always isolated from pork.

Table 1.2.*Campylobacter* isolates identified, sources of isolation and originof the samples.

Identification number	Food type	Origin	Species	Place of collection
CJ 1	Chiken carcass	Naples	*C.jejuni*	Poultry slaughterhouse
CJ3	Chiken carcass	Naples	*C.jejuni*	Poultry slaughterhouse
CJ5	Raw chiken	Naples	*C.jejuni*	Poultry slaughterhouse
CJ6	Raw chiken	Naples	*C.jejuni*	Poultry slaughterhouse
CJ7	Raw chiken	Naples	*C.jejuni*	Poultry slaughterhouse
CJ9	Chiken skin	Naples	*C.jejuni*	Poultry slaughterhouse
CJ10	Chiken skin	Naples	*C.jejuni*	Poultry slaughterhouse
CJ11	Raw chiken	Melito	*C.jejuni*	Poultry slaughterhouse
CJ12	Spicy chicken	Marano	*C.jejuni*	Butcher's shop
CJ13	Raw chiken	Naples	*C.jejuni*	Butcher's shop
CJ14	Raw chiken	Melito	*C.jejuni*	Butcher's shop
CJ15	Raw chiken	Gragnano	*C.jejuni*	Butcher's shop
CJ16	Raw chiken	Casoria	*C.jejuni*	Mini market
Cc17	Raw chiken	Naples	*C.coli*	Mini market
Cc18	Raw chiken	Naples	*C.coli*	Slaughterhouse
Cc19	Raw chiken	Naples	*C.coli*	Slaughterhouse
Cc20	Raw chiken	Naples	*C.coli*	Mini market
Cc21	Raw pork	Naples	*C.coli*	Slaughterhouse
Cc22	Raw pork	Naples	*C.coli*	Slaughterhouse
Cc23	Raw pork	Naples	*C.coli*	Slaughterhouse
Cc24	Chiken carcass	Naples	*C.coli*	Poultry slaughterhouse
Cc25	Chiken carcass	Naples	*C.coli*	Poultry slaughterhouse
Cc26	Chiken carcass	Naples	*C.coli*	Poultry slaughterhouse
Cc27	Raw chiken	Giugliano	*C.coli*	Mini market
Cc28	Chiken carcass	Naples	*C.coli*	Poultry slaughterhouse

CHAPTER 2. Comparative genomics of *Campylobacter* spp.

1. Introduction

Next-generation sequencing (NGS) refers to the set of technologies for sequencing nucleic acids that can sequence millions of DNA fragments in parallel.

Unlike earlier sequencing techniques, in which it was possible to analyze only a limited portion of the genome, sequencing of the whole genome offers a complete vision of the entire genomic sequence; this enables us to detect variants of single nucleotides, insertions/deletions, modifications of copy number and large structural variants. Thanks to recent technological innovations, the latest sequencers can sequence the whole genome more efficiently than ever.

Whatever type of equipment is used, DNA sequencing follows the same three steps: preparation of the sample to be analyzed, physical sequencing, and reassembly. Sample preparation consists of splitting the genome into several fragments of limited length; according to the method adopted, these fragments can be amplified in various ways. In the sequencing phase, each base in each fragment is identified, and reads are created. In reassembly, bioinformatics software is exploited in order to concatenate overlapping reads, thereby extending the length of the fragments. The greater the length of the sequence, the better the results will be, in that we have truer data to work on. Indeed, many applications require reads to be as long as possible, in order to yield precise results. The organizational scheme of

NGS can be divided into two macro-blocks: one pertaining to biology and one to bioinformatics. In the former, the genome is sequenced by identifying the nucleotides that make up the fragments (Adenine, Guanine, Cytosine and Thymine), thereby obtaining strings of characters. In the latter, these sequences are analyzed in order to join them, whenever possible, discard them, or correct them in the event of errors; in this way, the software user is provided with valid results, with the least amount of superfluous information. The technology is based on the analysis of the light emitted by each nucleotide, which enables its type to be identified. Unfortunately, however, the light emitted by the nucleotides is too faint; the fragments therefore have to be amplified by means of polymerase chain reaction (PCR), a technique that enables fragments of nucleic acid to be multiplied, and hence amplified.

2. Materials and Methods

2.1 Illumina Sequencing

Within the national network of the Istituti Zooprofilattici Sperimentali (IZS), the Teramo branch houses a center of excellence, the “Genomic Sequences” National Center, a reference center for the genomic sequencing of pathogens. Here, the 25 strains of *Campylobacter* isolated during the present research were sequenced. The equipment used was the new NextSeq500 for massive parallel NGS; this highly

versatile, innovative technology enables high-performance sequencing of DNA and RNA.

The NextSeq500 instrument has a maximum productivity of 120 Gb and enables up to 800 million sequences of nucleic acids to be sequenced simultaneously in each run in an average time of 24 hours.

Togeteher with tha NextSeq 500 System, the Illumina platform and the Nextera DNA XT Library Preparation Kit were used. This latter integrates the phases of DNA extraction, fragmentation, library preparation and library normalization, thereby providing the fastest and most flexible workflow in the portfolio of Illumina library preparation.

2.2 Downloading *Campylobacter* spp. genomes from databases

The analysis included 3055 genomes of *C. coli* and *C. jejuni* available in the NCBI RefSeq database. Files containing the assembled sequences (GCA) were downloaded. For these genomes, the available meta-data were curatedmanually. Specifically, the source of isolation was obtained from the NCBI or, if not available, from the reference publication. The data on the source of isolation were then grouped by host Table 2.1.

Table 2.1. Genomes downloaded from NCBI and reported sources of isolation

Source	Total strains	% total	N° *C.jejuni*	% *C.jejuni*	N° *C.coli*	% *C.coli*
Human	1097	35.9%	860	43.5%	237	22.0%
Chicken	580	19.0%	298	15.1%	282	26.2%
Wild birds	272	8.9%	242	12.2%	30	2.8%
Natural environment	249	8.2%	78	3.9%	171	15.9%
Stool	210	6.9%	195	9.9%	15	1.4%
Farm environment	218	7.1%	26	1.3%	192	17.8%
Not available	95	3.1%	83	4.2%	12	1.1%
Bovine	116	3.8%	82	4.1%	34	3.2%
Non-Human Primate	66	2.2%	40	2.0%	26	2.4%
Swine	57	1.9%	9	0.5%	48	4.5%
Turkey	37	1.2%	9	0.5%	28	2.6%
Milk and cream	47	1.5%	46	2.3%	1	0.1%
Goat and sheep	7	0.2%	7	0.4%	0	0.0%
Calf Liver	1	0.0%	1	0.1%	0	0.0%
Rabbit	1	0.0%	1	0.1%	0	0.0%
Mouse	1	0.0%	1	0.1%	0	0.0%
Dogs	1	0.0%	0	0.0%	1	0.1%
Total *Campylobacter* spp.	**3055**		**1978**		**1076**	

2.2 Bioinformatics analysis

The sequences obtained were filtered by means of the Prinseq software, In order to eliminate potentially erroneous bases due to sequencing errors. The reads of each genome were then assembled in contigs by means of the SPADES software; only contigs > 1,000 bp were maintained for the subsequent analyses. The genes were predicted by means of GeneMark. The analysis focused on the genes of virulence and antibiotic resistance by using the TORMES (https://github.com/nmquijada/tormes) pipeline; this uses the CARD database and the Virulence Factors Database to identify the genes of antibiotic resistance and virulence factors, respectively.One-hundred twenty-six virulence genes were identified in *Campylobacter jejuni* (Figure 2.1) and 111 in *Campylobacter coli* (Figure 2.2). The antibiotic resistance genes detected were respectively 33 in *Campylobacter jejuni* (Figure 2.3) and 27 in *Campylobacter coli* (Figure 2.4).

Phylogenetic trees were obtained by means of RAxML (https://cme.h-its.org/exelixis/web/software/raxml/) and were visualized in iTOL(https://itol.embl.de).

Figure 2.1 Heatmap showing the presence/absence of virulence genes in 1,978*Campylobacter jejuni* genomes. Colored bar indicates the source of isolation.

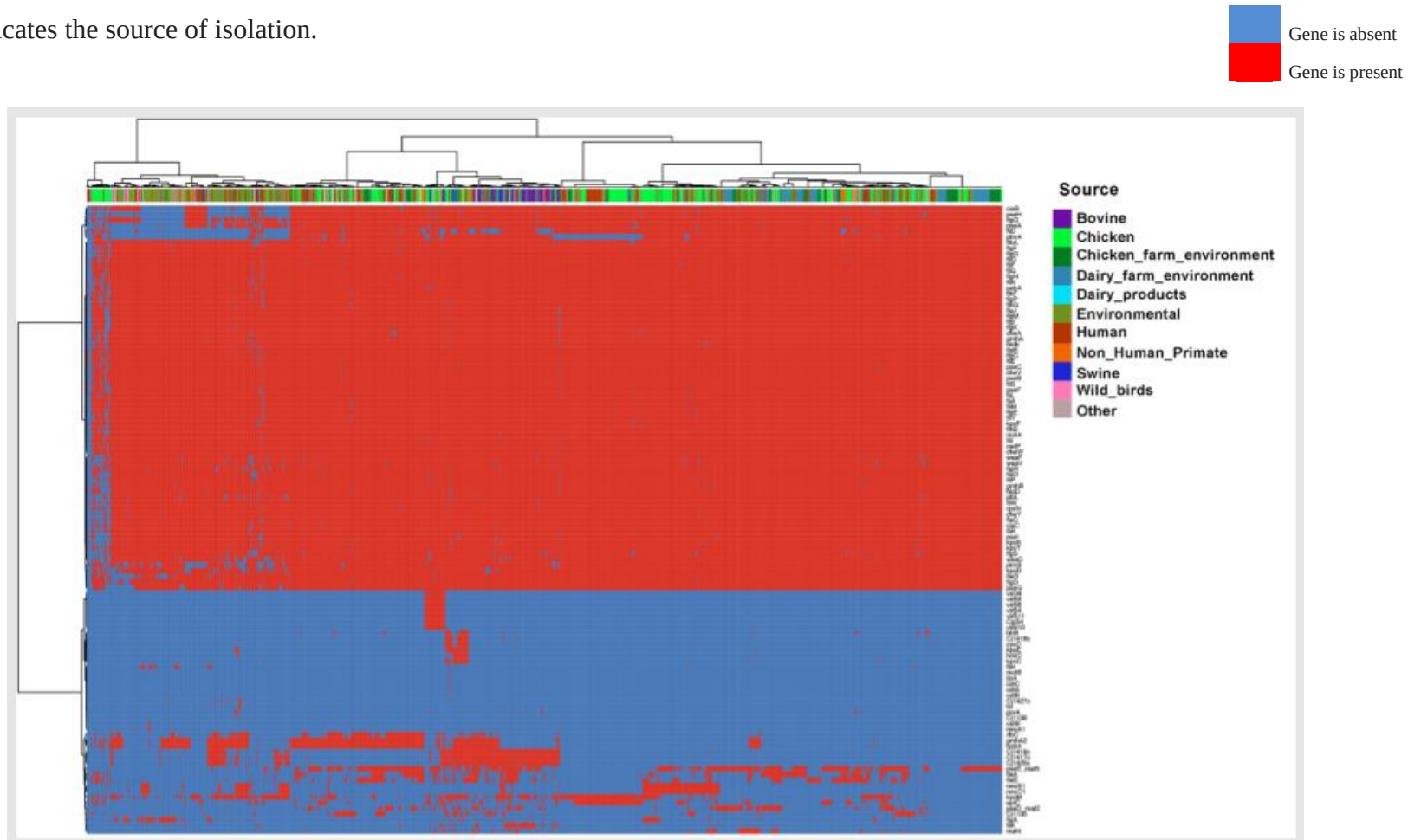


Figure 2.2 Heatmap showing the presence/absence of virulence genes in 1,076 *Campylobacter coli* genomes. Colored bar indicates the source of isolation.

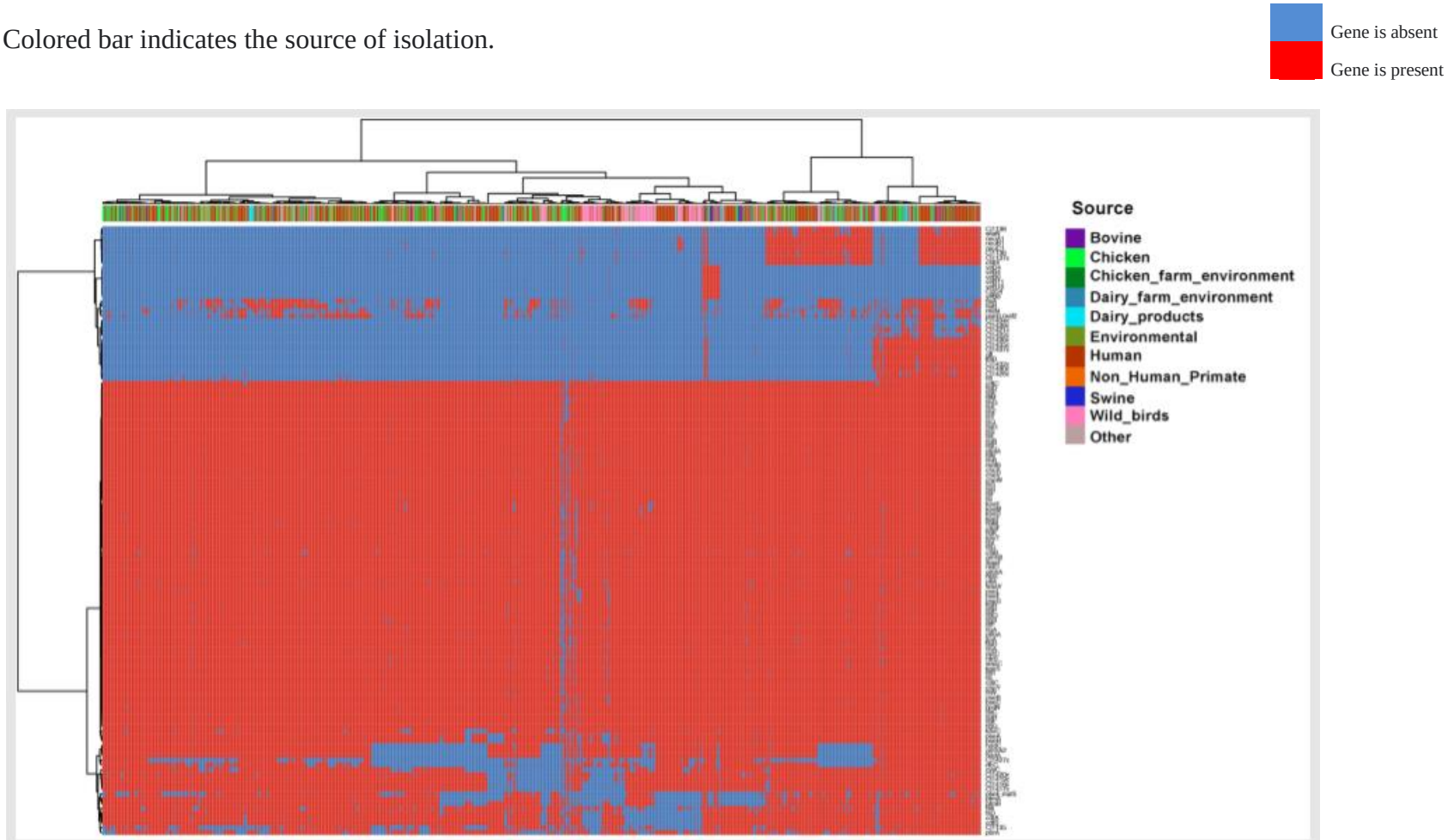


Figure 2.3 Heatmap showing the presence/absence of antibiotic resistance genes in 1,978 *Campylobacter jejuni* genomes.Colored bar indicates the source of isolation.

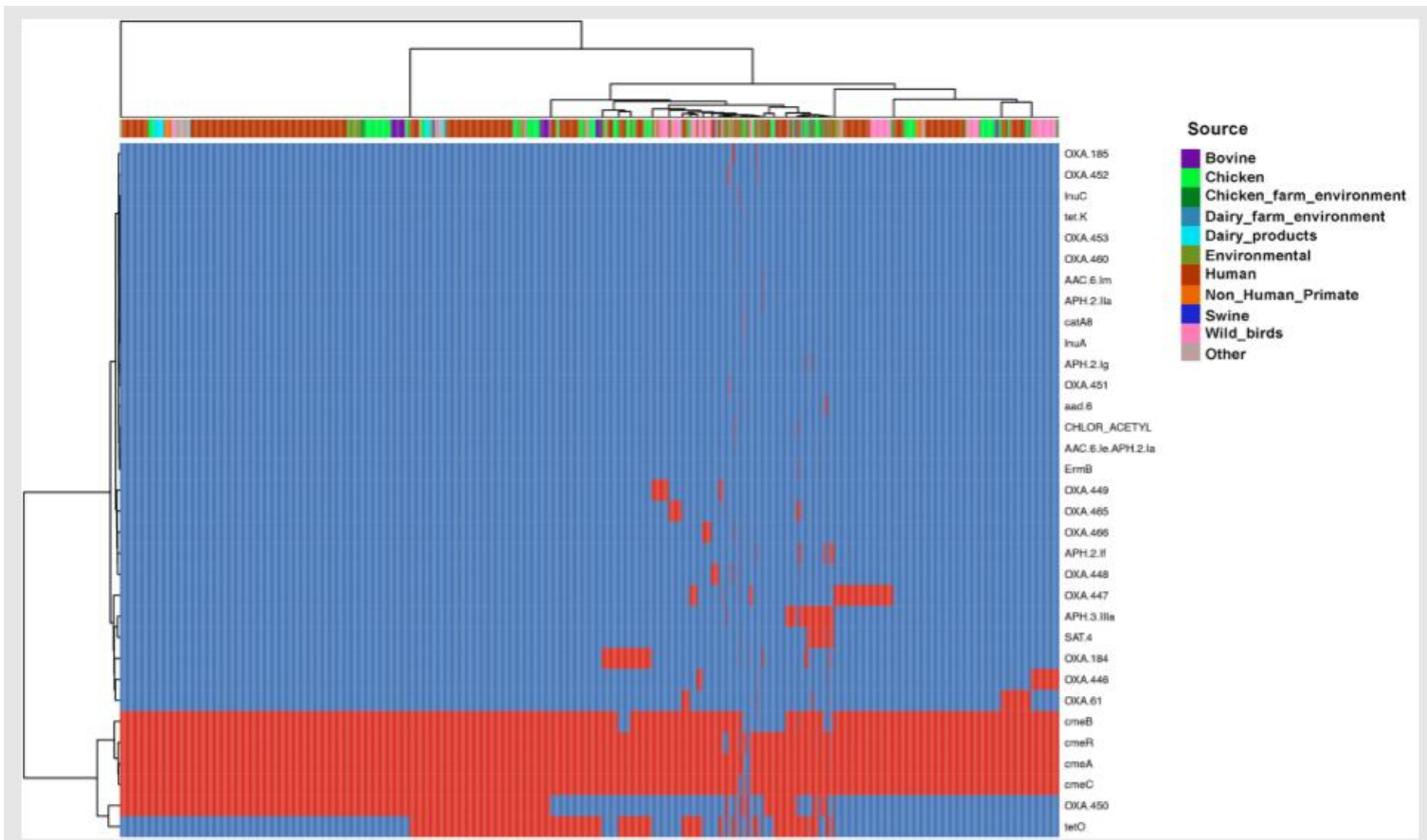
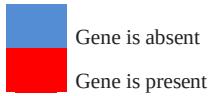
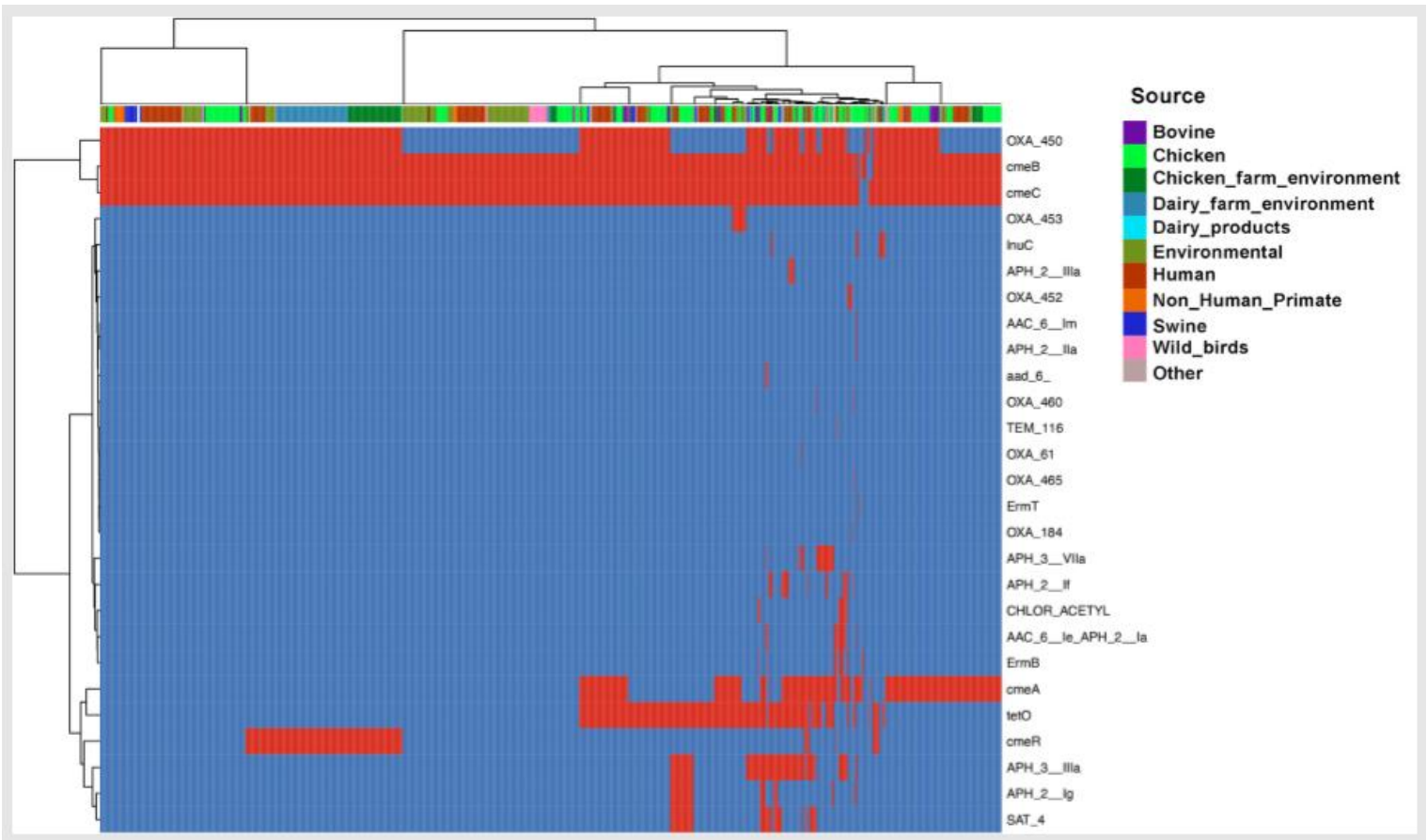


Figure 2.4 Heatmap showing the presence/absence of antibiotic resistance genes in 1,076 *Campylobacter coli* genomes. Colored bar indicates the source of isolation.





2.4 Reconstruction of *Campylobacter* spp. genomes from meta-genomes

From public databases, 2300 meta-genomes were also downloaded; these came from chicken intestines (n=1274), pig intestines (n=632 and urban waste water (n=234)The meta-genomes were assembled by means of the Megahit software, and the Metagenomics-Assembled Genomes (MAGs) were reconstructed by usingMetaBAT and filtering out the genomes with <80% completeness. MAGs were screened for the presence of *C. coli* and *C. jejuni*and these were integrated into the analysis, together with 7 MAGs (6 *C.jejuni*and 1 *C.coli*) previously reconstructed from meta-genomes from the human intestines (Pasolli et al., 2019). A total of24 (*C.jejuni*) and 27 (*C.coli*)MAGs from chicken intestines,, while no genome of *Campylobacter* spp. was found in the urban waste water and swine..

3. Results and discussion

From the NCBI, a total of 3,055 genomes (1,076 *C.coli* and 1,978 *C. jejuni*) were retrieved.

The 35.9% of available *Campylobacter* genomes come from human isolates, with *C. jejuni* representing the 43,5%. The second most important isolation source was the chicken gut and meat (19%),*C. coli (26,2%)*and *C. jejuni (15,1%)* genomes. Among genomes from wild birds and bovine, *C. jejuni* represented the 12,2% and 4,1%, respectively.

As for *C. coli* genomes, they come from farm environment (17,8%), natural environment /15,9%),swine (4,5%),turkey (2,6%).

The phylogenetic trees of each species were visualized in iTOL (Interactive Tree Of Life), an online tool for the visualization, annotation and management of phylogenetic trees.

Figure 2.5 shows the phylogenetic tree obtained for the genomes of *Campylobacter coli*; in the outer ring, the sources of isolation available in the NCBI are reported. It can be seen that 4 principal groups are formed, 2 of which gather strains isolated from environmental, suggesting the presence of subspecies that are not reported in the taxonomy. Indeed, unlike the case of *C. jejuni*, different subspecies of *C. coli* are not reported. In both species, a high degree of similarity can be seen among the genome of strains isolated from chicken, poultry farmsand human feces, indicating that human campylobacteriosis is mainly caused by strains from chicken origin. The new strains isolated fell into these two groups; the other two groups seem to comprise strains that are not transferable to humans: one comprising*Campylobacter coli* isolates mainly from cattle and from livestock farms in general, and the other comprising strains isolated from the environment and from wild birds.

Figure 2.5. Phylogenetic tree of *C. coli* genomes available in public databases. The most internal ring indicates the source of isolation, the most outer ring indicates the four sub-species identified

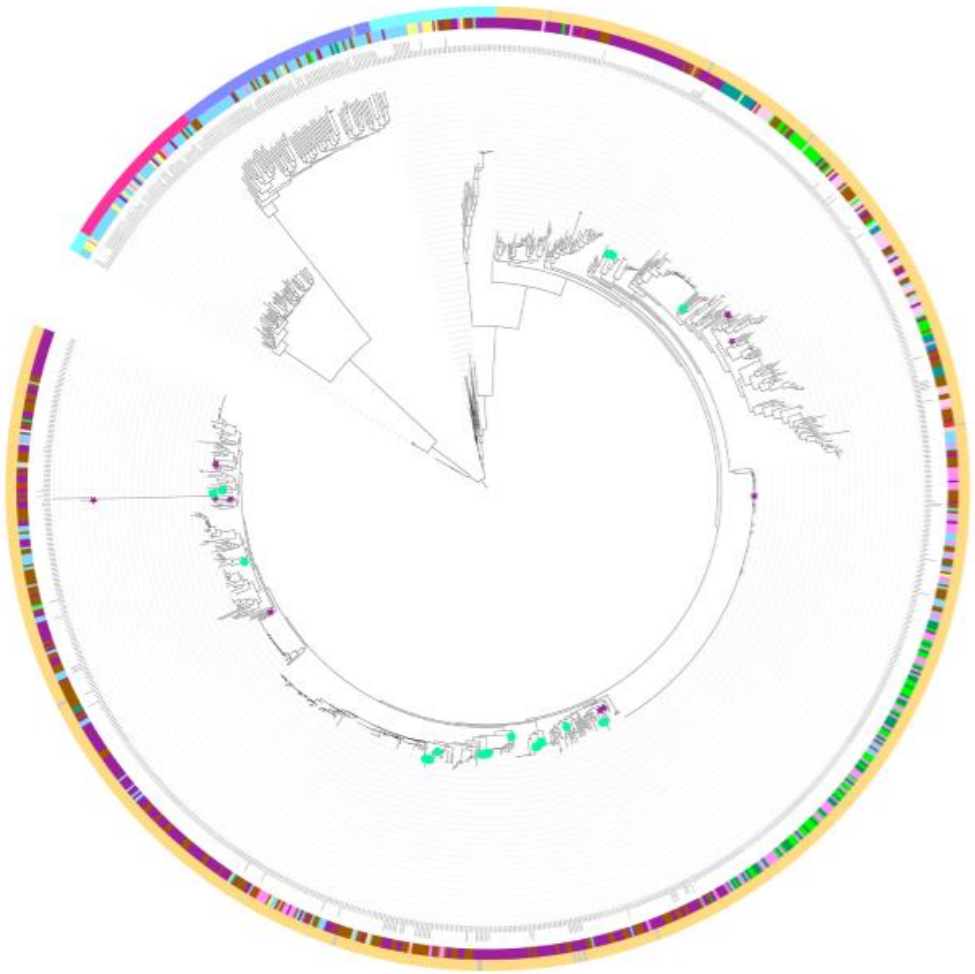
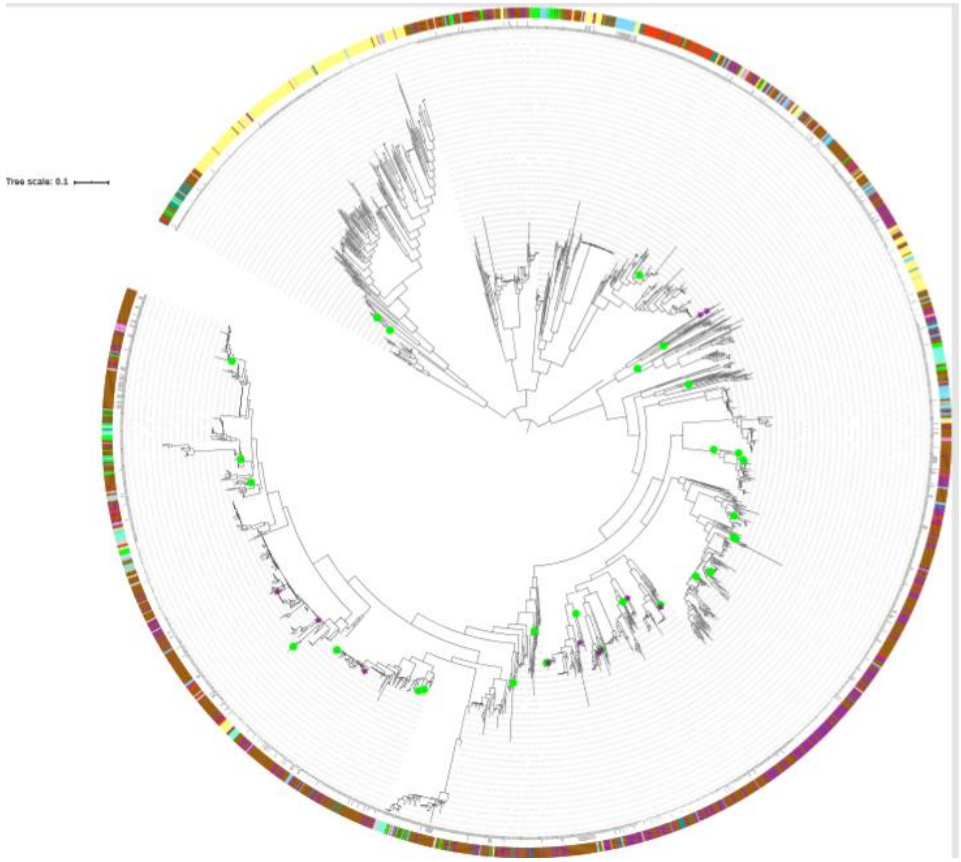


Figure 2.6 shows the phylogenetic tree obtained for the genomes of *Campylobacter jejuni*; in the outer ring, the sources of isolation available in the NCBI are reported. Here, three distinct groups can be seen, one of which is more clearly separate and is chiefly made up of genomes isolated from wild birds. The other two groups are similar in terms of the sources of isolation, with strains from chicken, poultry farms and from humans, isolated both from feces and from blood. The new strains isolated fit into these two groups. Although the taxonomy reports only two subspecies of *C. jejuni* (*C. jejuni* sub. *jejuni* and *C. jejuni* sub. *doylei*), these results suggest the presence of at least three distinct groups.The separation of genomes as a function of the host can also be observed in the MDS obtained on the basis of the MASH distance between each pair of genomes (Figures 2.7 and 2.8).

Figure2.6 Fylogenetic tree of *C. jejuni* genomes available in public databases. The outer ring indicates the source of isolation.



wild_bird
Primate
chicken
swine
human_stool
bovine
NA
human_patient_blood
environmental
dairy_products
chicken_farm_environment
dairy_farm_environment

Figure2.7. Non-Metric Multidimentional Scaling (nMDS) of *Campylobacter jejuni* genomes based on MASH distance matrix. Points (genomes) are coloured according to the isolation sources. MAGs reconstructed in this study are highlighted.

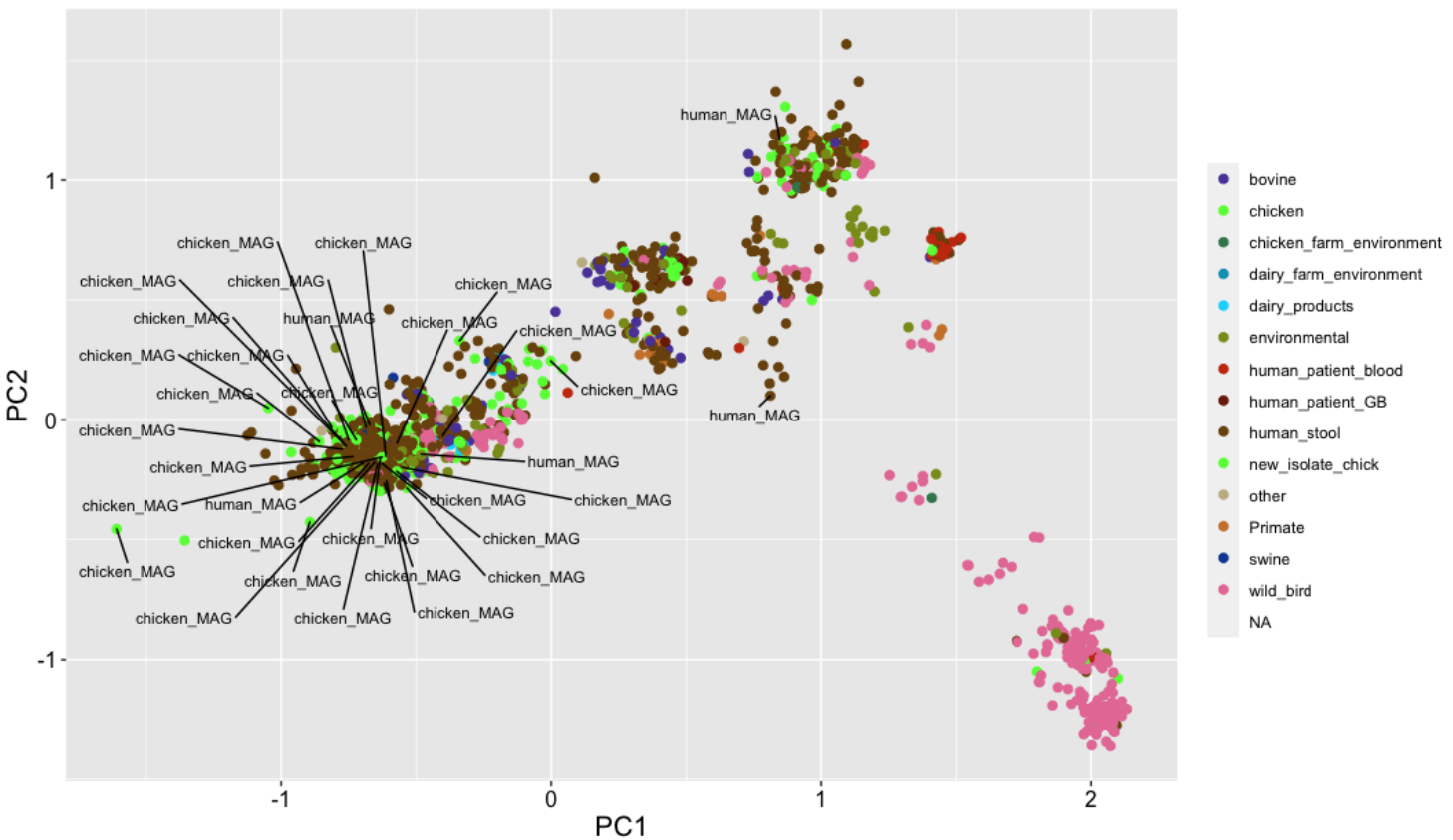
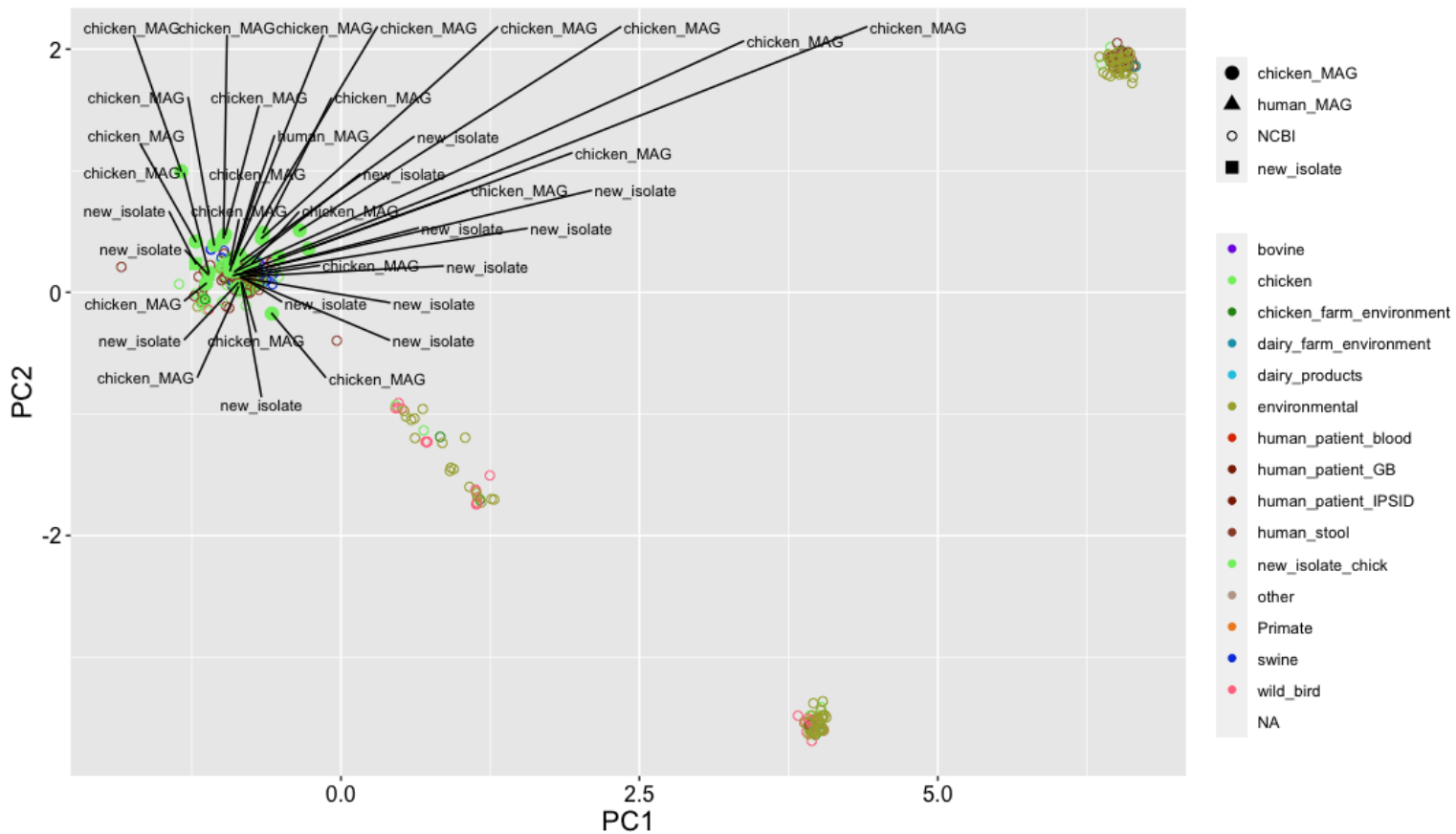


Figure 2.8 Non-Metric Multidimentional Scaling (nMDS) of *Campylobacter coli* genomes based on MASH distance matrix. Points (genomes) are coloured according to the isolation sources. MAGs reconstructed in this study are highlighted.



CHAPTER 3. Genomic screening virulence factors and antibiotic resistance, phenotypic testing for antibiotic resistance

1. Introduction

Antibiotics can be classified on the basis of their biological target, for example of their ability to inhibit cell wall synthesis (penicillin and cephalosporin), to disrupt the lipid structure of the cell wall (polymyxins), to inhibit protein synthesis by acting on the smaller (30s) ribosomal subunit (tetracycline and the aminoglycosides, including gentamicin) or the larger (50s) subunit (chloramphenicol and the macrolides), to inhibit the synthesis of nucleic acids by acting on the duplication of DNA (novobiocin) or on RNA transcription (rifamycins). According to their effects on microorganisms, antibacterials can be divided into: bacteriostatic antibiotics – those that block the growth of the bacterium, thereby facilitating its elimination by the organism; and bactericidal antibiotics, which cause the death of the bacterium. To ascertain whether an antibiotic is bacteriostatic or bactericidal, we determine the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC). The MIC is the minimum concentration of antibiotic able to prevent the development of microorganisms (μg/ml). The MBC is the minimum concentration of antibiotic able to cause the death of the bacterial cells (μg/ml). If the antibiotic is bactericidal, the values of MIC and MBC coincide. If the antibiotic is bacteriostatic, the values of MIC and MBC are different (MBC>MIC). Some antibiotics are defined as "broad-

spectrum", in that they act on both Gram-positive and Gram-negative bacteria; other antibiotics have a narrow spectrum, as they act only on specific bacteria. In the case of many microbial species that have the ability to mutate frequently, it is often necessary to combine several antibiotics in order to tackle resistant microorganisms, and sometimes to prevent their appearance. Some molecules are improperly called antibiotics, in that they are not of natural origin, but synthesized; these are defined as chemotherapeutic agents.

Chemotherapeutic agents include the sulfonamides and trimethoprim, which act by inhibiting the synthesis of the folates, indispensable substrates for the formation of nucleotides and amino acids. Quinolones, which are chemotherapeutic agents derived from nalidixic acid, also act by inhibiting topoisomerase II; this protein, also known as gyrase, is made up of two subunits, A and B, which allow unwinding and rewinding of the bacterial DNA. Subunit A cuts the DNA at specific sites, while subunit B acts on the so-called negative supercoiling of the DNA. The quinolones act by inhibiting subunit A of gyrase, and hence the replication of the bacterial DNA (novobiocin, by contrast, acts on subunit B, and can therefore exert a synergic action with the quinolones). Recent years have seen a marked increase in the multidrug resistance (MDR) acquired as a result of the role played by the efflux pumps in expelling antimicrobial drugs; indeed, transmembrane transport proteins constitute the points of entry and exit of various molecules, ions and nutrients to/from the cell.

The study of these membrane transport processes is essential to our understanding of their impact on microbial pathogenesis.

1.1 Classification of natural and synthetic antibiotics

The fluoroquinolones (e.g. ciprofloxacin, enrofloxacin, etc.) are a family of synthetic broad-spectrum antibacterial agents that act against a wide range of Gram-positive and Gram-negative organisms (Appelbaumet al., 2000; Hooper,1998). They are currently among the drugs of choice for the treatment of campylobacteriosis in humans and of other bacterial diseases in both humans and animals (Redgrave et al., 2014). The fluoroquinolones target two essential enzymes, DNA gyrase and topoisomerase IV, preventing DNA replication (Hooper, 1998). Generally speaking, theresistance of bacteria to fluoroquinolones stems from mutations in the genes that code for the subunits of DNA gyrase (GyrA and GyrB), topoisomerase IV (ParC and ParE), or both (Payot S. et al., 2002). In *Campylobacter*, the chief mechanism of resistance to the fluoroquinolones is mediated by point mutations in the region of GyrA that determines resistance to quinolones (Payot et al., 2006). To date, GyrB mutations have not been implicated in fluoroquinolone resistance in *Campylobacter* (Bachoual et al., 2001). In addition to GyrA mutations, the functional multidrug efflux pump, CmeABC, is also required for fluoroquinolone resistance in *Campylobacter* (Luo et al., 2003).

The macrolide antibiotics (azithromycin, clarithromycin, erythromycin, telithromycin, etc.) are a class of drugs used to treat gastric diseases caused by *Campylobacter* and respiratory tract infections in humans (Chu, 1999). The macrolides target the 50S subunit of the bacterial ribosomal and inhibit protein synthesis. Bacterial resistance to the macrolides is generally mediated by three mechanisms: enzymatic in activation of the macrolides, modification or point mutations in the target, and increased drug efflux (Gibrel A et al., 2006). A recently identified rRNA methylation enzyme is ErmB (Deng et al., 2015); this gene alone is able to confer a high level of resistance to the macrolides (Qin et al., 2014). It is noteworthy that the ErmB gene is associated to multidrug resistant genomic islands (MDRGI), which include several resistance genes (aacA-aphD, sat4, aphA-3, fosXCC, aad9 and tetO); however, the presence of the cmeABC pump (Linet al., 2002) also influences multidrug resistance.

The tetracyclines exert a broad-spectrum action against Gram-positive and Gram-negative bacteria, chlamydia, mycoplasma, rickettsiae and parasitic protozoa (Chopra et al., 2001). As tetracyclines have been widely used for many years, a number of determinants of resistance to this class of drugs have been observed in a variety of bacteria (Roberts, 2005). Tetracycline resistance is generally mediated by one of four mechanisms: efflux pump, chemical modification of the tetracyclines, ribosomal protection proteins, and rRNA mutations (Connel SR et al., 2003). Tetracycline resistance in *Campylobacter* has been seen to be conferred by the

ribosomal protection protein TetO and by efflux pumps (CmeABC and CmeG) (Jeon et al., 2011). The CmeABC and CmeG efflux pumps contribute to both intrinsic and acquired resistance to tetracycline in *Campylobacter* (Gibrel et al., 2007). CmeABC works in synergy with TetO to confer a high level of tetracycline resistance (Lin et al., 2002).

The aminoglycosides are bactericidal antibiotics that bind to ribosomes and inhibit protein synthesis (Spahn et al., 1996). These antimicrobials are generally endowed with broad-spectrum bactericidal activity, and are used to treat acute and systemic *Campylobacter* infections (Lawrence et al., 2010), though their action is limited in anaerobic environments. The aacA4 gene codes for the aminoglycoside 6′-N-acetyltransferase, AAC (6′) - Ib7, which confers resistance to tobramycin, kanamycin and neomycin (Rather et al., 1992). The growing prevalence and the emergence of new genes of resistance to gentamicin have prompted an increasing number of studies on the mechanisms of resistance to this antibiotic. The gene coding for aminoglycoside 3-adenylyltransferase (aadA) confers resistance to streptomycin and spectinomycin, while the gene coding for aminoglycoside 6-adenylyltransferase (aadE) confers resistance only to streptomycin.

The β-lactams are a class of broad-spectrum antibiotics that inhibit the biosynthesis of the bacterial cell wall. Antibiotics of this class contain a beta-lactarin in their molecular structure. The β-lactams are the most widely used antibiotics, accounting for more than half of the global market of antibiotics (Hamad, 2010). In recent

decades, the prevalence of bacteria that are resistant to the β-lactams has increased markedly (Jovetic, 2010). To date, three mechanisms contributing to the resistance of *Campylobacter* to the β-lactams have been identified: enzymatic in activation, reduced absorption, and efflux pump (Lachance et al.,1991). The efflux pumps CmeABC and CmeDEF can also contribute to β-lactam resistance. Indeed, inactivation of these efflux pumps determines greater susceptibility to ampicillin (Akibaet al., 2006). OXA61 (Cj0299) is the only β-lactam that has been identified and characterized in *C. jejuni* (Zeng et al, 2014).

Fosfomycin is a broad-spectrum antibiotic that exerts bactericidal action against Gram-positive and Gram-negative bacteria (Forsegreen et al., 1983). Fosfomycin inhibits the synthesis of the bacterial cell wall by inactivating the enzyme essential for the catalysis of the biosynthesis of the bacterial peptidoglycan (Fillgrove et al., 2003). In *Campylobacter*, fosfomycin resistance is rare and of low level (Shwainger K. et al., 2008). To date, the only mechanism of resistance to fosfomycin identified in *Campylobacter* is the fosXCC gene; this is contained in the MDRGI of *C. coli* and can be transferred to *C. jejuni* by means of natural transformation (Wang et al., 2015).

The non-fluorinated (chloramphenicol) or fluorinated (florfenicol) phenicols are highly effective against a broad range of Gram-positive and Gram-negative bacteria. At one time, the phenicols were widely used in human and veterinary medicine for the prevention and treatment of many bacterial infections. In

Campylobacter, resistance to the phenicols is mediated through enzymatic inactivation by chloramphenicol acetyltransferase, mutations of the target site in 23SrRNA, alteration of the target elements in 23SrRNA by means of rRNA methyltransferase Cfr (C) or enhanced extrusion by means of efflux pumps. Moreover, the recently identified RE-CmeABC variant of the multidrug efflux pump can, in itself, confer a high degree of phenicol resistance (Yao et al., 2016).

Arsenic compounds have often been used in the poultry industry to control diseases and to promote growth. However, owing to the potential risk to human health and the environment, they have recently been withdrawn from use in poultry in the United States. Nevertheless, the organic form of arsenic, roxarsone, is still used as an additive to poultry feed in other countries. To survive in the setting of poultry production, *Campylobacter* has developed ways of resisting the action of arsenic compounds. Indeed, *Campylobacter* isolates from conventional poultry products have displayed significantly higher levels of resistance than those from products from poultry untreated with antimicrobials (Sapkota et al., 2006). Recently, various mechanisms of arsenic disintoxication have been identified in *C. jejuni*, including arsenate reductase ArsC, the transporters of arsenic efflux Acr3 and ArsB, and methylarsenite efflux permease ArsP (Chen J et al., 2015).

The presence of the operon containing acr3 is significantly associated with a high level of resistance to arsenite and arsenatein *Campylobacter*. Furthermore, inactivation of acr3leadstoreductionsintheMICsofbotharseniteand arsenate. Acr3 is

not involved in the resistance to other classes of antibiotics in *Campylobacter* (Wang et al., 2009).

In addition to resistance to single antibiotics, the phenomenon of multidrug resistance (MDR) must be considered. MDR may be intrinsic or acquired by cells exposed to a drug; these cells display a heightened ability to expel not only one drug, but also several drugs that are not correlated structurally and functionally. This mechanism is implemented by efflux pumps, which play an essential role in the intrinsic and acquired resistance to structurally different antimicrobials. In *Campylobacter*, several multidrug efflux pumps (CmeABC, CmeDEF and CmeG) have been characterized from the functional standpoint in terms of their contributions to antibiotic resistance (Jeon et al., 2011).

The cmeABC efflux pump is coded for by an operon that comprises three genes: cmeA, cmeB and cmeC. It consists of a membrane fusion protein (CmeA), an internal membrane transporter (CmeB) and an external membrane protein (CmeC). CmeABC expels toxic compounds and contributes to the resistance of *Campylobacter* to structurally different antimicrobials (Pumbwe et al., 2002), such as macrolides and fluoroquinolones (Cagliero et al., 2005). This system of efflux has been functionally characterized in*C. jejuni* (Lin et al., 2005) and *C. coli* (Cagliero et al., 2005).

In general, the other efflux pump, CmeDEF, seems to play a minor, and strain-dependent, role in antibiotic resistance, and its natural function in the physiology of *Campylobacter* remains partly unknown (Akibaet al., 2006).

The study of antibiotic resistance in *Campylobacter jejuni* and *Campylobacter coli* is regulated by EU Decision 2013/652/UE. This lays down detailed rules for standard monitoring and for the reports on antimicrobial resistance (AMR) presented by the EU member states, in conformity with article 7, paragraph 3, article 9, paragraph 1, annexe II, part B, and annexe IV of the Directive 2003/99/CE. Monitoring and reporting concern the strains of *Campylobacter* obtained from samples of specific foodstuffs and of specific populations of animals used for food production. Six antimicrobials are tested: erythromycin, ciprofloxacin, tetracycline, gentamicin, nalidixic acid and streptomycin;isolates are deemed susceptible or resistant on the basis of the cut-off values established by the European Committee on Antimicrobial Susceptibility Testing (http://www.eucast.org), as reported in Table 3.1. MDR is defined as simultaneous resistance to a minimum of three drugs (Maesaar et al., 2016). The genes of antibiotic resistance were sought in the genomes of the strains isolated, and identified by means of the CARD database. Table 3.2 reports the classes of antibiotics to which they belong and the genes that determine resistance. In this study, other genes of interest were also sought, in addition to those that determine resistance to the single antibiotics for which the MIC was determined.

Table 3.1 Panel of antimicrobial substances to be included in AMR monitoring, EUCAST interpretative thresholds for resistance and concentrationranges to be tested in *C.jejuni* and *C.coli*

Antimicrobial	Species	Interpretative thresholds of AMR (mg/L)		Range of concentrations (mg/L) (No of wells in brakets)
		ECOFF (a)	Clinical breakpoint (b)	
Erytromycin	*C.jejuni*	> 4	> 4	1-128 (8)
	C.coli	> 8	> 8	
Ciprofloxacin	*C.jejuni*	> 0,5	> 0,5	0,12-16 (8)
	C.coli	> 0,5	> 0,5	
Tetracycline	*C.jejuni*	> 1	> 1	0,5-64 (8)
	C.coli	> 2	> 2	
Gentamicin	*C.jejuni*	> 2	NA	0,12-16 (8)
	C.coli	> 2	NA	
Nalidixic acid	*C.jejuni*	> 16	NA	1-64 (8)
	C.coli	> 16	NA	
Streptomycin (c)	*C.jejuni*	> 4	NA	0,25-16 (8)
	C.coli	> 4	NA	

(a) EUCAST epidemiological cut-off values

(b) EUCAST clinical resistence breakpoints

(c) At a voluntary basis

NA: not available.

Table 3.2. Genes involved in antibiotic resistance, grouped by antibiotic.

Antibiotic molecules	Antibiotic classes	Gene detected
Tetracycline	Tetracycline	tetO
Ciprofloxacyne	Quinolones	cmeA, cmeB,cmeC,cmeR,
Nalidixico acid	Quinolones	cmeA, cmeB,cmeC,cmeR
Erytromycin	Macrolides	cmeA, cmeB,cmeC,cmeR,ErmB
Gentamicin	Aminoglycosides	aad(6),AAC(6)-Im,AAC(6')-le-APH(2")-la,APH(2")-if,APH(2")-Ig,APH(2")-IIa,APH(2")-IIIa,APH(3')-IIIa,APH(3')-VIIa
Streptomicyn	Aminoglycosides	
Lincomicyn	Lincosamides	InuC,ErmB,ErmT
Nucleosides	Nucleosides	SAT-4
Beta-lactams	Cephalosporin;penam	oxa450,oxa184,oxa449,oxa466,oxa61,TEM-116
Roxarsone	Arsenical-resistance protein ACR3	acr3
Multidrug and bile resistance	Cephalosporin;fluoroquinolone;fusidic acid;macrolide	cmeA,cmeB,cmeC,cmeR, cmeD,cmeE, cmeF
Phenicoli	Cloroanphenicles	Campylobacter_coli_chloramphenicol_acetyltransferase(cloracetyl)

2. Materialsand Methods

2.1 Determination of MIC

Antibiotic resistance was evaluated on the basis of the MIC, i.e. the lowest concentration of the antibiotic able to inhibit the visible growth of the microorganism considered. This method is based on the ability of the microorganism to produce visible growth in micro-titration plate wells ofbroth containing serial dilutions of antimicrobial agents. Accordingly, an appropriate dilution in Sensititre Mueller-Hinton broth of the microorganism under investigation (0.5 McFarland - $1x10^5$UFC/ml), measured by means of a Sensititre Nephelometer (Thermo Scientific), was added to the wells of a plate (Sensititre EUCAMP 2, Thermo Scientific) containing increasing concentrations of the antimicrobial agents. After incubation of the plate in conditions suited to the growth of the microorganism, the MIC was read by means of the Sensititre Vizion system and SWIN software. Growth appears as turbidity or as a deposit of cells on the bottom of the well; the MIC is recorded as the lowest concentration of the antimicrobial agent that inhibits visible growth.

2.2 Screening the genomes for the presence of antibiotic-resistance genes

The genes detected are reported in the two tables (Table 3.3 and Table 3.4) below. The genomes were screened for antibiotic-resistance and virulence genes using the TORMES pipeline and the CARD and Virulence Factor Databases, respectively

Table 3.3. Virulence-related genes detected

Virulence gene	Mechanism of action	Reference
flA, flhB,flgB,flgE,fliM,fliY	Motility	Karlyshev et al, 2002
flaA,flaB,flaC,fliA,rpoN,flag, flha	Motility	Ghorbanalizadgan et al,2014
cheA, cheB,cheR,cheW,cheY	Chemotacticfactors	Sanchez et al, 2019
cadF,dnaJ,pdlA,racR,capA virB11, ILVE,peb1A,MOMP/porA,	Adhesion	de Olivera et al, 2018
kpsM,kpsE,pgld,waaF,peb4, pgp1	Capsula	Hammed et al, 2020
iamA,ciaB,ceuE,ciaC, ciaD, ciaI	Invasione	Hkoolman et al, 2016
cdtA,cdtB,cdtC	Citotoxicfactors	Asakura et al, 2008
katA,sodB,ppk1, spoT, cj1556, csrA, cosR, cprS e nuoK,ppk1, ppk2, cstA, spoT, hspR, htrA, htrB, sodB, katA, perR, ahpC, dnaJ, cosR, cprR, cprS, nuoK	Stress response and survival	Tegtmeyer et al, 2021
wlaN	GBS (GuillanBarrè)	Endtz et al, 2000

Table3.4. Antibiotic-resistance genes detected

Antibiotic resistance gene		Reference
tet (O),tet(K)	Tetracyclin	Cantero et al, 2017
cmeA, cmeB, cmeC,cmeR,cmeDEF	Quinolones	Cantero et al, 2017
APH(3')-VIIa APH(3')-IIIa APH(2")-If APH(2")-Ig APH(2")-IIa APH(2")-IIIa AAC(6')-Ie-APH(2")-Ia aad(6) AAC(6')-Im	Aminoglycosides	Llarena 2017
InuC,ErmB,ErmT	Lincosamides	Liu et al, 2017
SAT-4	Nucleosides	Du et al, 2018
OXA61,OXA184,OXA 450,OXA452,OXA453, OXA449,OXA460,OXA465, OXA446,OXA466,OXA185, OXA448,OXA449,OXA451, OXA447,TEM-116	Betalattamici	Proietti et al,*2020*
ErmB,ErmT	Macrolided	Shen et al, 2017
Campylobacter_coli_chloramphenicol_acetyltransferase	Fenicol	Wang et al, 1990

3. Results and discussion

3.1 MIC determination in the new isolates

For each strain tested, the result is expressed as a numerical value that represents the lowest concentration at which no visible growth of the bacterium occurs, followed by the related interpretative category: S (susceptible), R (resistant), I (intermediate), according to the ECOFF for *Campylobacter* specified in EU Directive 2013/652/EU of 14 November, 2013 (Table 3.5). The notations S, R and I are assigned on the basis of the specific breakpoints.

Table 3.5. Evaluation of the antibiotic resistance of the 25 strains isolated.

Strain identification number		Source	Ciprofloxacin	Erythromycin	Gentamicin	Nalidixic Acid	Streptomycin	Tetracycline
Cj1	DS9977114	carcass of chicken						
Cj3	DS9977113	carcass of chicken						
Cj9	DS9977112	chicken skin						
Cj10	DS9977118	chicken skin						
Cj11	DS9977111	chicken						
Cj14	DS9977119	chicken						
Cj5	DS9977133	chicken						
Cc6	DS9977129	chicken						
Cj7	DS9977122	chicken						
Cj12	DS9977128	chicken						
Cj13	DS9977131	chicken						
Cj15	DS9977127	chicken						
Cj16	DS9977123	chicken						
Cc21	DS9977110	pig						
Cc22	DS9977121	pig						
Cc23	DS9977120	pig						
Cc24	DS9977115	carcass of chicken						
Cc26	DS9977117	carcass of chicken						
Cc28	DS9977116	carcass of chicken						
Cc17	DS9977124	chicken						
Cc18	DS9977125	chicken skin						
Cc19	DS9977126	carcass of chicken						
Cc20	DS9977132	chicken						
Cc27	DS9977130	chicken						
Cj25	DS9977109	carcass of chicken						

Resistant
Sensible

Of the 25 strains analyzed, 16 proved resistant to ciprofloxacin, 14 to nalidixic acid, 12 to tetracycline, 2 to streptomycin, and only 1 to gentamicin; no strain displayed resistance to erythromycin. Resistance to at least one class of antibiotic was recorded in 12 (92%) of 13 strains of *C. jejuni*, and in 7 (58%) of 12 strains of

C. coli. Multidrug resistance was found in about 30.7% and 41.7% of strains of *C. jejuni* and *C. coli*, respectively.

According to the 2020 EFSA data from monitoring in Italy, *C. coli* strains are the most resistant in percentage terms. In the present study, while *C. jejuni* displayed the greatest resistance to single antibiotics, *C. coli* displayed the highest percentage of multidrug resistance. Specifically, *C. coli* cc21 isolated from pork proved to be resistant to four antibiotics. The presence of *Campylobacter* isolates displaying combined resistance tociprofloxacin and erythromycin is of great importance to public health, since both compounds are classed as CIA (*Critically Important Antimicrobials*) for the treatment of*Campylobacter* infections in humans (WHO, 2019). The Italian data (EFSA, 2020) report 24% of combined resistance. However, none of the new strains isolated proved resistant to both of these compounds.

3.2 Genotype-phenotype correlation

One of the objectives of the present study was to seek a correlation between the phenotypic and genotypic data obtained, in order to better understand the mechanisms that influence the antimicrobial resistance of *Campylobacter jejuni* and *C. coli*. Tables 3.6 and 3.7 show the correlations between the data on the MIC and the presence, in the genomes sequenced, of specific genes capable (or not) of determining antimicrobial resistance in each species.

Table 3.6 Values of MIC (minimum inhibitory concentration) for each *Campylobacter jejuni* isolate and indication of the antibiotic-resistance genes detected. ND, gene not detected.

Antimicrobico		***Cj1***	***Cj3***	***Cj5***	***Cj7***	***Cj9***	***Cj10***	***Cj11***	***Cj12***	***Cj13***	***Cj14***	***Cj15***	***Cj16***	***Cj25***
Ciprofloxacin	MIC	16	0,5	≤0,12	4	8	8	16	16	8	16	16	8	≤0,12
	Detected gene	cmeA,cmeB,cmeC,cmeR	cmeA,cmeC,cmeR	cmeA,cmeB,cmeC,cmeR	cmeA,cmeB,cmeC,cmeR	cmeA,cmeB,cmeC,cmeR	cmeA,cmeC,cmeR	cmeA,cmeB,cmeC,cmeR	cmeA,cmeC,cmeR	cmeA,cmeB,cmeC,cmeR	cmeA,cmeB,cmeC,cmeR	cmeA,cmeB,cmeC,cmeR	cmeA,cmeB,cmeC,cmeR	cmeA,cmeB,cmeC,cmeR
Erythromycin	MIC	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1
	Detected gene	cmeA,cmeB,cmeC,cmeR	cmeA,cmeC,cmeR	cmeA,cmeB,cmeC,cmeR	cmeA,cmeB,cmeC,cmeR	cmeA,cmeB,cmeC,cmeR	cmeA,cmeC,cmeR	cmeA,cmeB,cmeC,cmeR	cmeA,cmeC,cmeR	cmeA,cmeB,cmeC,cmeR	cmeA,cmeB,cmeC,cmeR	cmeA,cmeB,cmeC,cmeR	cmeA,cmeB,cmeC,cmeR	cmeA,cmeB,cmeC,cmeR
Gentamicin	MIC	0,25	1	≤0,12	≤0,12	0,25	0,5	0,25	0,25	0,25	0,25	0,5	0,25	0,25
	Detected gene	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Nalidixic Acid	MIC	>64	8	4	16	64	>64	64	>64	64	8	>64	>64	8
	Detected gene	cmeA,cmeB,cmeC,cmeR	cmeA,cmeC,cmeR	cmeA,cmeB,cmeC,cmeR	cmeA,cmeB,cmeC,cmeR	cmeA,cmeB,cmeC,cmeR	cmeA,cmeC,cmeR	cmeA,cmeB,cmeC,cmeR	cmeA,cmeC,cmeR	cmeA,cmeB,cmeC,cmeR	cmeA,cmeB,cmeC,cmeR	cmeA,cmeB,cmeC,cmeR	cmeA,cmeB,cmeC,cmeR	cmeA,cmeB,cmeC,cmeR
Streptomycin	MIC	1	2	0,5	0,5	1	2	1	1	1	1	2	1	1
	Detected gene	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Tetracycline	MIC	≤0,5	32	≤0,5	≤0,5	≤0,5	>64	≤0,5	32	≤0,5	32	1	64	4
	Detected gene	ND	TetO	ND	TetO	ND	TetO	ND	TetO	ND	TetO	ND	TetO	ND

Table 3.7 Values of MIC (minimum inhibitory concentration) for each *Campylobacter coli* isolate and indication of the antibiotic-resistance genes detected. ND, gene not detected.

Antimicrobico		***Cc6***	***Cc17***	***Cc18***	***Cc19***	***Cc20***	***Cc21***	***Cc22***	***Cc23***	***Cc24***	***Cc26***	***Cc27***	***Cc28***
Ciprofloxacin	MIC	8	≤0,12	≤0,12	≤0,12	≤0,12	1	≤0,12	≤0,12	16	8	8	16
	Detected gene	cmeB,cmeC,cmeR	cmeB,cmeC	cmeB,cmeC	cmeB,cmeC	cmeB,cmeC	cmeA,cmeB,cmeC	cmeB,cmeC	cmeA,cmeB,cmeC	cmeA,cmeB,cmeC	cmeA,cmeB,cmeC	cmeA,cmeB,cmeC	cmeA,cmeB,cmeC
Erythromycin	MIC	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	4	≤1	≤1	≤1
	Detected gene	cmeB,cmeC,cmeR	cmeB,cmeC	cmeB,cmeC	cmeB,cmeC	cmeB,cmeC	cmeA,cmeB,cmeC,cmeD,cmeE	cmeB,cmeC	cmeA,cmeB,cmeC	cmeA,cmeB,cmeC	cmeA,cmeB,cmeC	cmeA,cmeB,cmeC	cmeA,cmeB,cmeC
Gentamicin	MIC	0,5	0,5	0,5	0,5	0,5	4	0,5	0,5	1	0,5	0,5	0,5
	Detected gene	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Nalidixic Acid	MIC	64	4	4	4	8	>64	8	8	>64	>64	>64	>64
	Detected gene	cmeB,cmeC,cmeR	cmeB,cmeC	cmeB,cmeC	cmeB,cmeC	cmeB,cmeC	cmeA,cmeB,cmeC	cmeB,cmeC	cmeA,cmeB,cmeC	cmeA,cmeB,cmeC	cmeA,cmeB,cmeC	cmeA,cmeB,cmeC	cmeA,cmeB,cmeC
Streptomycin	MIC	1	2	2	2	2	16	4	>16	2	2	1	2
	Detected gene	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Tetracycline	MIC	>64	≤0,5	≤0,5	≤0,5	≤0,5	≤0,5	≤0,5	4	>64	64	>64	32
	Detected gene	Tet(O)	ND	ND	ND	ND	ND	ND	Tet(O)	Tet(O)	Tet(O)	Tet(O)	Tet(O)

Two findings are of the greatest interest. In both species, no strain displayed resistance to erythromycin *in vitro*; however, sequencing of the whole genome revealed, in all the strains analyzed, the presence of some genes that potentially confer this resistance: *cmeA, cmeB, cmeC*and *cmeR.*

By contrast, on MIC determination, only one strain, *C. coli* Cc21, displayed resistance to gentamicin, while no gene that might indicate specific resistance to this antibiotic was found in the genome of the strain. It is plausible that, in this specific case, the resistance encountered was due to multiple resistance generated by the presence of a set of genes expressed.

Table 3.8 reports the presence of genes that endow *Campylobacter* spp with resistance or multiple resistance to other antibiotics not evaluated by means of MIC.

Table 3.8 Occurrence of genes conferring resistance to new antimicrobial agents in the genomes of the 25 newly isolated strains.

Antibiotic molecules			Gene detected	Antibiotic classes
	N°	%		
Lincomicin	0	0	InuC,ErmB,ErmT	Lincosamides
Nucleosides	0	0	SAT-4	Nucleosides
Beta-lactams	22	88	oxa450,oxa184,oxa449,oxa466,oxa61,TEM-116	Cephalosporin;penam
Roxarson	17	68	acr3	Arsenical-resistance protein ACR3
Multidrug and bile resistance	25	100	cmeA,cmeB,cmeC,cmeR,cmeD,cmeE,cmeF	Cephalosporin;fluoroquinolone;fusidic acid;macrolide
Phenicol	0	0	Campylobacter_coli_chloramphenicol_acetyltransferase(cloracetyl)	Cloroanfenicol

As can be seen, the efflux pumps detected in all the strains analyzed play a key role in the physiology of *Campylobacter*, as they confer intrinsic and acquired resistance to several toxic compounds, such as bile salts, antibiotics and various detergents (Lin et al., 2002). Although *Campylobacter* is a major enteric pathogen, and despite the progress made in recent years in understanding its complicated and multifactorial pathogenesis, there is a gap in our understanding of the combination of phenotypic and genotypic characteristics.

Moreover, it is probable that new mechanisms of antibiotic resistance in *Campylobacter* will continue to emerge. Innovative strategies are therefore needed in order to curb the increase and diffusion of antibiotic-resistant strains of *Campylobacter*.

3.3 Screening for antibiotic-resistance genes in the genomes of *C. coli* and *C. jejuni*

A total of 1,115 genomesof*Campylobacter coli* downloaed from NCBI were screened for the presence of genes involved in antibiotic resistance. Table 3.9 shows that only the genes *cmeB* and *cmeC*, which are responsible for resistance to quinolones and which influence efflux pumps, were detected in 100% of the genomes; the genes that influence other efflux pumps, *cmeDEF*, were not detected in *Campylobacter coli.*

Among the genes that confer resistance to the beta-lactams, oxa450 was found in 748 genomes, i.e. 68%. By contrast, the genes oxa184, oxa449 and oxa446 were never found. The genes oxa460 and oxa465 were detected only in three strains isolated from chicken.

The genes that confer resistance to the aminoglycosides do not seem to be common in the genomes of *C. coli*.Indeed, only the gene APH(3')-IIIa was found in about 13% of the genomes.

Resistance to tetracycline, which is expressed through the gene tet(O), was recorded in 392 (35%) of the 1,115 strains.

The gene Tem116 was present in only one strain, isolated from human feces.

Table 3.9. Prevalence (%) of antibiotic-resistance genes in the genomes of *C. coli* present in public databases

GENE	No. of *Campylobacter coli* genomes in which it is present	**%**
cmeA	336	30,7
cmeB	1093	100,0
cmeC	1092	99,9
CmeR	211	19,3
cmeD	0	0,0
cmeE	0	0,0
cmeF	0	0,0
cmeR	0	0,0
oxa 184	0	0,0
oxa 450	748	68,4
oxa 452	6	0,5
oxa 453	17	1,6
oxa 449	0	0,0
oxa 460	1	0,1
oxa 465	1	0,1
oxa 61	2	0,2
oxa 446	0	0,0
tetO	392	35,9
acr3	0	0,0
APH(3')-VIIa	45	4,1
APH(3')-IIIa	143	13,1
APH(2'')-If	27	2,5
APH(2'')-Ig	50	4,6
APH(2'')-IIa	1	0,1
APH(2'')-IIIa	7	0,6
AAC(6')-Ie-APH(2'')-Ia	18	1,6
AAC(6')-Im	1	0,1
SAT-4	71	6,5
TEM-116	1	0,1
ErmT	1	0,1

ErmB	14	1,3
lnuC	17	1,6
aad(6)	3	0,3
Campylobacter_coli_chloramphenicol_acetyltransferase	13	1,2

The genes involved in antibiotic resistance were also sought in 2,014 genomes of *Campylobacter jejuni*.Table 3.10 shows that *C. jejuni* displays a greater diffusion of antibiotic resistance than *C. coli*.Specifically, an additional five genes that confer resistance to the beta-lactams (oxa185, oxa448, oxa449, oxa451, oxa447) were detected. Moreover, also in the case of *C. jejuni*, oxa450 was the gene detected in the greatest number of strains, about 60%.

The genes *cmeABC*, which express resistance to the quinolones through the efflux pumps, were found in many genomes of *C. jejuni*; indeed, in over 93%.

The genes that confer resistance to the lincosamides and macrolides were rarely detected. ErmT was never found, while ErmB was present in only three strains isolated from human feces, pork and chicken meat.

The gene tet(O), which confers resistance to the tetracyclines, was detected in 828 of 2,014 genomes, i.e. over 41%.

The genes of antibiotic resistance to the macrolides and the aminoglycosides proved to be rare.

Table 3.10 Prevalence of antibiotic-resistance genes in the genomes of *C. jejuni* present in public databases

GENE	Number of *Campylobacterjejuni* genomes in which it is present	**%**
cmeA	2020	100,0
cmeB	1890	93,6
cmeC	2018	99,9
CmeR	2005	99,3
cmeD	0	0,0
cmeE	0	0,0
cmeF	0	0,0
oxa 184	153	7,6
oxa 450	1227	60,7
oxa 452	6	0,3
oxa 453	1	0,0
oxa 449	49	2,4
oxa 460	1	0,0
oxa 465	39	1,9
oxa 61	117	5,8
oxa 446	74	3,7
oxa466	23	1,1
oxa 185	7	0,3
oxa 448	28	1,4
oxa 449	49	2,4
oxa 451	3	0,1
oxa447	155	7,7
aad(6)	7	0,3
tetO	828	41,0
catA8	2	0,1
APH(3')-VIIa	0	0,0
APH(3')-IIIa	138	6,8
APH(2'')-If	25	1,2
APH(2'')-Ig	5	0,2
APH(2'')-IIa	2	0,1
APH(2'')-IIIa	0	0,0
AAC(6')-Ie-APH(2'')-Ia	2	0,1
AAC(6')-Im	1	0,0
SAT-4	71	3,5
TEM-116	0	0,0
ErmT	0	0,0
ErmB	3	0,1
InuA	6	0,3
lnuC	2	0,1

tet(K)	1	0,1
Campylobacter_coli_chloramphenicol_acetyltransferase	4	0,2

3.4 Screening for virulence factor-related genes in the genomes of *C. coli* and *C. jejuni.*

In the genomes of *C. coli*, the most common virulence factor-related genes were those responsible for the mechanisms of motility, invasion and adhesion (Table 3.11).

Only one genome, GCA_000686425 isolated from human feces, was seen to possess the genes *cdtA, cdtB* and *cdtC*, which trigger cytotoxic factors; the strain GCA_000686425 again isolated from human, was the only one that presented the genes *motB* and*JlpA*, which are responsible for motility and invasion.

Moreover, it can be seen that the genes responsible for chemotactic factors (*cheA, cheV, cheW)* and those responsible for adhesion, invasion and motility (*cadF*, *flgE*, *motA*) were detected in 100% of the genomes analyzed.

Some genes involved in the development of biofilm were also found in fairly high percentages: i.e. the genes responsible for cell motility(*flaA, flaB, flaC, flaG, fliA, fliS* and *flhA*) and the gene that acts on the cell surface (*waaF*).

The percentages of the occurrence of the two genes *flaA* and *flaB*, which code for two respective flagellins, were almost identical. This highlights the capacity for adhesion and invasion of the strains involved, in that we know that, if the former gene is suppressed, the motility of the microorganism, and consequently its capacity for penetration, is reduced (Vandamme et al,2005; Silva et al, 2011).

In none of the genomes of *C. coli* we detected the gene *wlaN*, which is responsible for Guillain-Barré syndrome.

Table 3.11 Prevalence of virulence factor-related genes in the genomes of *C. coli.*

Virulence gene	N°. of *Campylobacter coli* genomes in which it is present	**%**	**Mechanism of action**
cadF	1115	100,0%	Adhesion
jlpA	1	0,1%	
pebA	1103	98,9%	
porA	11	1,0%	
pseA	960	86,1%	
pseB	1110	99,6%	
pseC	1106	99,2%	
virB10	25	2,2%	
virB11	25	2,2%	
virB4	25	2,2%	
virB8	25	2,2%	
virB9	25	2,2%	
virD4	24	2,2%	
ciaB	953	85,5%	Invasion
ciaC	1097	98,4%	
pflA	1079	96,8%	
ptmA	753	67,5%	
flaA	272	24,4%	Invasion andAdhesion
flaB	293	26,3%	
flaC	1099	98,6%	
flaD	1089	97,7%	
flaG	1051	94,3%	
flgA	94	8,4%	
flgB	1107	99,3%	
flgC	1110	99,6%	
flgD	1092	97,9%	
flgE	1115	100,0%	
flgF	1103	98,9%	
flgG	1106	99,2%	
flgH	1108	99,4%	
flgI	1111	99,6%	
flgJ	1114	99,9%	
flgK	1108	99,4%	
flgM	1113	99,8%	
flhA	1094	98,1%	
flhB	1115	100,0%	
flhF	1109	99,5%	
maf4	93	8,3%	Motility

motA	1115	100,0%	
motB	1	0,1%	
flhG	1109	99,5%	
fliA	1115	100,0%	
fliD	811	72,7%	
fliE	1111	99,6%	
fliF	1115	100,0%	
fliG	1115	100,0%	
fliH	24	2,2%	
fliI	1113	99,8%	
fliK	59	5,3%	
fliL	1107	99,3%	
fliM	1113	99,8%	
fliN	1107	99,3%	
fliP	1096	98,3%	
fliQ	1108	99,4%	
fliR	1102	98,8%	
fliS	1109	99,5%	
fliY	1108	99,4%	
fliW	1099	98,6%	
rpoN	1101	98,7%	
pseD/maf2	238	21,3%	
pseE/maf5	522	46,8%	
rpoN	1101	98,7%	
flgP	1110	99,6%	
flgQ	1009	90,5%	
flgR	1098	98,5%	
flgS	1062	95,2%	
eptC	205	18,4%	
pseG	1052	94,3%	
pseF	1108	99,4%	
pseI	1091	97,8%	
pseH	903	81,0%	
ptmB	1010	90,6%	
Cj1416c	25	2,2%	Capsule
Cj1417c	204	18,3%	
Cj1419c	193	17,3%	
Cj1420c	197	17,7%	
Cj1427c	8	0,7%	
Cjp54	25	2,2%	
fcl	4	0,4%	
gmhA2	372	33,4%	
hddA	369	33,1%	
hddC	23	2,1%	

kpsC	26	2,3%	
kpsD	1058	94,9%	
kpsE	33	3,0%	
kpsF	1113	99,8%	
kpsM	425	38,1%	
kpsS	1088	97,6%	
kpsT	1104	99,0%	
rfbC	188	16,9%	
rfbC	188	16,9%	
Cj1135	122	10,9%	Los (lipooligosaccharide)
Cj1136	1	0,1%	
cstIII	2	0,2%	
gmhA	1108	99,4%	
waaC	1067	95,7%	
waaF	1091	97,8%	
waaV	1091	97,8%	
hldD	1104	99,0%	
hldE	1105	99,1%	
htrB	34	3,0%	
neuA1	4	0,4%	
neuB1	159	14,3%	
neuC1	151	13,5%	
cdtA	1	0,1%	Citotoxicfactors
cdtB	1	0,1%	
cdtC	1	0,1%	
cheA	1115	100,0%	Chemotacticfactors
cheV	1115	100,0%	
cheW	1115	100,0%	
cheY	1095	98,2%	

With regard to *Campylobacter jejuni*, also in this case, the genes most commonly detected were those involved in the mechanisms of motility, invasion and adhesion (Table 3.12).

Unlike the case of *C. coli*, the genes that determine cytotoxic factors (*cdtA, cdtB, cdtC*) were detected in >85% of the genomes. As in *C. coli*, the genes influencing chemotaxis as a virulence factor (*cheA*, *cheV*, *cheW*, *cheY*) were found in 100% of the genomes.

We found 343 C. jejuni strains (17%) that presented the gene WlaN, which is responsible for Guillain-Barré syndrome (GBS). In the literature, there are no data that demonstrate that C. coli is also a possible cause of GBS (Belkum et al., 2009). Many recent studies have highlighted the scant knowledge and shortage of genomic studies of the LOS(lipooligosaccharide)of C. coli, and a few studies(Kolehmainen et al., 2019) have tried to extend knowledge of the ecology ofC. coli, of its pathogenesis and of the host-pathogen interaction.

As in the case of *Campylobacter coli*, some virulence genes involved in the development of biofilm were detected in fairly high percentages. The same is true of the genes responsible for cell motility *(flaA, flaB, flaC, flaG, fliA, fliS* and *flhA*) and the gene that acts on the cell surface (*waaF*). In this case, the occurrence of the genes *flaA* and *flaB* were very similar, thereby demonstrating a marked adhesive and invasive capacity.

Table 3.12 Prevalence of virulence factor-related genes in the genomes of *C. jejuni* present in public databases.

Virulence gene	N° of *Campylobacter jejuni* genomes in which it is present	%	Mechanism of action
cdtA	1728	85,8%	Citotoxicfactors
cdtB	1749	86,8%	
cdtC	1994	99,0%	
cheA	2014	100,0%	Chemotacticfactors
cheV	2014	100,0%	
cheW	2014	100,0%	
cheY	2014	100,0%	
cadF	2009	99,8%	Adhesion
jlpA	1990	98,8%	
pebA	2011	99,9%	
porA	1529	75,9%	
pseA	1912	94,9%	
pseB	2007	99,7%	
pseC	2006	99,6%	
virB10	45	2,2%	
virB11	45	2,2%	
virB4	44	2,2%	
virB8	46	2,3%	
virB9	44	2,2%	
virD4	44	2,2%	
flaA	699	34,7%	Invasion and Adhesion
flaB	649	32,2%	
flaC	2012	99,9%	
flaD	1994	99,0%	
flaG	2021	100,3%	
flgA	2003	99,5%	
flgB	2014	100,0%	
flgC	2014	100,0%	

flgD	2014	100,0%	
flgE	2014	100,0%	
flgF	2014	100,0%	
flgG	2014	100,0%	
flgH	2014	100,0%	
flgI	2014	100,0%	
flgJ	2014	100,0%	
flgK	2007	99,7%	
flgM	2008	99,7%	
flhA	2005	99,6%	
flhB	2014	100,0%	
flhF	2014	100,0%	
ciaB	1941	96,4%	
ciaC	2014	100,0%	Invasion
pflA	1981	98,4%	
ptmA	1461	72,5%	
flgP	2014	100,0%	
flgQ	2005	99,6%	
flgR	2004	99,5%	
flgS	1995	99,1%	
eptC	1998	99,2%	
flhG	2013	100,0%	
fliA	2014	100,0%	
fliD	1717	85,3%	
fliE	2014	100,0%	
fliF	2014	100,0%	
fliG	2014	100,0%	Motility
fliH	2014	100,0%	
fliI	2014	100,0%	
fliK	1670	82,9%	
fliL	2013	100,0%	
fliM	2011	99,9%	
fliN	2014	100,0%	
fliP	2004	99,5%	
fliQ	2014	100,0%	
fliR	2008	99,7%	
fliS	2014	100,0%	
fliW	2010	99,8%	

fliY	2012	99,9%	
glf	259	12,9%	
pseD/maf2	680	33,8%	
pseE/maf5	1137	56,5%	
pseF	2004	99,5%	
rpoN	2007	99,7%	
kpsT	2013	100,0%	Motility, Adhesion and Invasion
maf4	501	24,9%	
motA	2014	100,0%	
motB	2014	100,0%	
pseG	2011	99,9%	Flagella
pseH	1907	94,7%	
ptmB	1537	76,3%	
cysC	1529	75,9%	Capsule
fcl	304	15,1%	
gmhA2	1535	76,2%	
hddA	1534	76,2%	
hddC	1749	86,8%	
kfiD	250	12,4%	
kpsC	1857	92,2%	
kpsD	1998	99,2%	
kpsE	1983	98,5%	
kpsF	2000	99,3%	
kpsM	1992	98,9%	
kpsS	2002	99,4%	
rfbC	1240	61,6%	
Cj1416c	1756	87,2%	
Cj1417c	1753	87,0%	
Cj1419c	1789	88,8%	
Cj1420c	1729	85,8%	
Cj1421c	147	7,3%	
Cj1422c	115	5,7%	
Cj1426c	226	11,2%	
Cj1427c	765	38,0%	
Cj1432c	240	11,9%	
Cj1434c	60	3,0%	
Cj1435c	255	12,7%	
Cj1436c	246	12,2%	

Cj1437c	251	12,5%	
Cj1438c	91	4,5%	
Cj1440c	238	11,8%	
Cjp54	46	2,3%	
Cj1135	1299	64,5%	
Cj1136	405	20,1%	
Cj1137c	396	19,7%	
Cj1138	325	16,1%	
cstIII	395	19,6%	
gmhA	2014	100,0%	
gmhB	1997	99,2%	
hldD	2009	99,8%	Los (lipooligosaccharide)
hldE	2010	99,8%	
htrB	2004	99,5%	
neuA1	413	20,5%	
neuB1	424	21,1%	
neuC1	415	20,6%	
waaC	2001	99,4%	
waaF	2001	99,4%	
waaV	1977	98,2%	
wlaN	343	17,0%	GuillanBarrè

GENERAL DISCUSSION

Campylobacter is the first cause of zoonosis in Europe, *C. coli* and *C.jejuni* species most transferable to humans come mainly from the consumption of inadequately cooked chicken meat and from poor domestic handling which often leads to cross-contamination between raw foods and cooked. Recent studies approach the quantification of *Campylobacter* contamination during food preparation (Yao Bai et al, 2020), highlighting the importance of timely and adequate cleaning to prevent cross-contamination and the need to educate the consumer about correct handling of food. foods to reduce the risk of foodborne infections.

The data from the latest EFSA report on antibiotic resistance (EFSA, 2020) indicate that *C. coli* is more resistant than *C. jejuni*; however, the genetic data reported in the tables of the sequences in NCBI seem to suggest the opposite. We can therefore deduce that, *in vitro*, the behavior of *Campylobacter*, and hence its resistance, may be conditioned not by the single gene; rather, it is clear that its pathogenicity is caused by a multifactorial multidrug resistance, and that the phenotypic and genotypic correlations are of fundamental importance to our understanding of the various factors of pathogenicity in *Campylobacter*.

CONCLUSIONS

Genomic sequencing is proving to be increasingly important to our understanding of the relationships between the phenotypic characteristics and the genotypic profile of *Campylobacter*; the future challenge will be to link these data together, in order to clarify the mechanisms that influence its pathogenicity.

It will also be important to understand how the genes that influence the formation of biofilm succeed in controlling and conditioning the persistence of *Campylobacter* in the environment and the role that this could play in the transmission of this pathogen.

It emerged from the present study that, in order to understand the virulence of *Campylobacter*, it is essential to explore the multiple factors of its pathogenicity. It is equally important to realise that the identification of the specific genes of resistance to antibiotics must necessarily be correlated with the presence of the efflux pumps that contribute to determining multidrug resistant. Indeed, the efflux pumps are among the main determinants of antibiotic resistance in microorganisms, in that they remove the antibiotic from the bacterial cell.

The study of efflux pump inhibitors is aimed at producing new compounds that have greater inhibitory activity and less cytotoxic activity.

In the future, the availability of efflux pump inhibitors will help to curb the spread of *Campylobacter* and to reduce its virulence. Indeed, efflux pump inhibitors can

dissipate the proton gradient or directly inhibit the efflux pump; to date, however (Hannula et al., 2008), it has been seen that efflux pump inhibitors and adjuvant compounds exert a strong selective pressure on microbial populations, facilitating the rapid emergence of resistant strains. Moreover, efflux pump inhibitors and adjuvant compounds are cytotoxic for the superior cells.

New studies (Atin Sharma et al 2019) are therefore being undertaken with a view to discovering natural inhibitors that can at least weaken the system of efflux pumps, thereby reducing multidrug resistance.

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Overall evaluation of the PhD thesis

PhD course in Food Science

Cycle XXXIII

REVIEWER (*first and last name*) ……Ilario Ferrocino……...…

AFFILIATION: Department of Agricultural, Forest and Food Sciences, DISAFA, University of Turin, Turin, Italy

PhD candidate (*first and last name***) SILVIA CASTELLANO**

Overall Evaluation of PhD thesis

The overall objective of the thesis was the isolation and genomic characterization of wild strains of *Campylobacter* sp. isolated in Italy and then the comparison with available genomes already publish with the aim to find the link between genomic and phenotipic feature. The thesis is divided in one preface that introduced the work and presented the objectives and 3 chapters.

Chapter 1 is dedicated to the isolation and identification of *Campylobacter* from poultry meat and pork from retail outlets and slaughterhouses.

Chapter 2 is mainly focused on comparative genomics of *Campylobacters* by NGS.

Chapter 3 is mainly focused on phenotypic testing for antibiotic resistance and virulence factors as well as the comparison between the genomic and the phenotypic feature.

There are few errors in the thesis that can be easily clarified. In general, the work is interesting and the methodology adequate.

In conclusion, Silvia's work can be considered sufficient and I am favorable to the defense of the thesis.

Therefore:

[X] The PhD candidate is admitted to the public defence of the thesis in the ordinary final exam session;

[ ] The PhD candidate is admitted to the public defence of the thesis **with postponement to the extraordinary session of the final exam***;

Date 17/06/21

Signature

* **the extraordinary session of the final exam** wil be set by June 30th 2021 (or with an extension, according to the **D.L.n. 34 of 19/05/2020, art. 236, comma 5)**

Overall evaluation of the PhD thesis

PhD course in Food Science

Cycle XXXIII

REVIEWER (*first and last name*) Beatriz Melero

AFFILIATION: University of Burgos

PhD candidate (*first and last name***)** Silvia Castellano

Overall Evaluation of PhD thesis The thesis presents well-defined objectives focused on the characterization of *Campylobacter* spp. strains at phenotypic and genomic level that will provide relevant information to the microbiological risk assessment. The introduction provided an extensive bibliographic revision, well referenced and deep, including basic aspects on the pathogen, disease produced, pathogenic factors and antibiotic resistance. The methodology used is advanced and the techniques used are adequate to achieve the objectives. Moreover, the results and conclusions demonstrate excellent performance.
The PhD candidate has a publication and has presented two research studies in national Conferences. Moreover, the scientific and technological knowledge have been improved attending specific courses covering a high number of hours.

Therefore:

[X] The PhD candidate is admitted to the public defence of the thesis in the ordinary final exam session;

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Signature

Beatriz Melero

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