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A STUDY OF CROSS-CONTAMINATION EVENTS OF *Campylobacter* spp. IN DOMESTIC KITCHENS ASSOCIATED WITH CONSUMER HANDLING PRACTICES OF RAW POULTRY

by

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Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa*
to fulfil the requirements of Master of Science degree in Applied Microbiology

by
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Abstract

Campylobacteriosis is the most reported zoonosis in the European Union since 2005 and the most common cause of bacterial foodborne diarrhoeal disease worldwide. In 2017, 596 cases of human campylobacteriosis were reported in Portugal. C*ampylobacter jejuni* is the main species infecting humans, but the observed prevalence of *C. coli* in Portugal is higher than that reported for other western countries. It has been recognized that contaminated chicken is the major vehicle for consumer's exposure to *Campylobacter*. This work was developed in the scope of SafeConsume project and its main objective was to evaluate possible cross-contamination events that can contribute to the spread of *Campylobacter* spp. in domestic kitchen environments during food preparation. Thus, 18 households were visited in October 2017 and the period between February and April 2018 to observe consumers preparing a recipe that included poultry and a raw vegetable salad. Poultry samples and swabs from domestic kitchen surfaces and utensils were collected before and after food preparation. Samples were also taken from tap handle, cabinet, drawer and refrigerator handles and the counter top surface. Other surfaces were sampled depending on observed behaviours during the individual food preparation sessions, such as: kitchen cloth, hand towel, sponge, cutting boards and the sink. Detection and enumeration of *Campylobacter* were performed according to the methods recommended by the International Organization for Standardization and species confirmation was performed by a multiplex Polymerase Chain Reaction assay. Pheno- and genotypic characterization of 72 *Campylobacter* spp. isolates was carried out through antimicrobial susceptibility, Pulse-Field Gel Electrophoresis (PFGE) and *flaA*-short variable region (SVR) sequencing. Of the 18 chicken samples analysed, 14 were *Campylobacter*-positive at least by one of the methods applied (occurrence of 77.8%). The microbial load ranged from $< 1.0 \times 10^1$ to 2.2×10^3 Colony Forming Units/g, with only one sample showing a contamination level above 10^3 CFU/g, the established limit present in Regulation (EC) No 2017/1495. Cross-contamination events were observed in four kitchens, between the chicken meat and two cutting boards, two sinks and one kitchen cloth. Both *C. jejuni* and *C. coli* were recovered from these surfaces/utensils. Very high levels of resistance to ciprofloxacin (100%) and tetracycline (94.4%) were observed. High resistance to erythromycin was also observed in this study (40.3%), differing from values reported by EFSA in 2016. *Campylobacter coli* isolates showed higher resistance to all antimicrobial agents tested than *C. jejuni*. Additionally, multidrug resistance (MDR) was observed in 63.9% of the isolates, of which 75.6% were *C. coli*. PFGE typing showed a high diversity among isolates, as well as *flaA*-SVR typing (29 pulsotypes, 16 *flaA* alleles and 8 *flaA* peptide identities). These results highlight the potential for the dissemination of resistant *Campylobacter* strains in the environment through the preparation of chicken meat and the need to educate the consumer for an appropriate handling of raw poultry meat products.

Keywords: *Campylobacter* ssp.; poultry; consumer practices, cross-contamination; campylobacteriosis

Resumo

A campilobacteriose é a zoonose mais reportada na União Europeia desde 2005 e a causa mais comum de diarreias de origem bacteriana transmitidas por alimentos em todo o mundo. Em 2017, foram comunicados 596 casos de campilobacteriose humana em Portugal, sendo *C. jejuni* a principal espécie a infetar humanos, embora a prevalência de *C. coli* em Portugal seja superior à relatada por outros países ocidentais. Os produtos avícolas contaminados são reconhecidos como um importante veículo para a exposição do consumidor a *Campylobacter*. Este trabalho foi desenvolvido no âmbito do projeto SafeConsume e teve como objetivo principal avaliar possíveis eventos de contaminação cruzada que contribuem para a disseminação de *Campylobacter* spp. no ambiente de cozinhas domésticas durante a preparação de alimentos. Assim, foram visitadas 18 casas em outubro de 2017 e entre fevereiro e abril de 2018 para observação dos consumidores durante a preparação de um prato com frango e de uma salada de vegetais crus. Foram retiradas amostras da cozinha antes e após a preparação dos alimentos. Os locais sujeitos a amostragem foram a torneira, os puxadores dos armários, das gavetas e do frigorífico assim como a bancada da cozinha. Foram também retiradas amostras de outras superfícies, dependendo dos comportamentos observados durante as sessões individuais de preparação de alimentos, por exemplo: pano de cozinha, pano das mãos, esponja, tábuas de corte e banca. A deteção e a enumeração de *Campylobacter* foram realizadas de acordo com os métodos recomendados pela International Organization for Standardization e a confirmação da espécie através de reacção em cadeia da polimerase no formato multiplex. A caracterização feno e genotípica de 72 isolados de *Campylobacter* spp. foi realizada através da suscetibilidade antimicrobiana, eletroforese em gel de campo pulsado (PFGE) e sequenciação da *short variable region* do gene *flaA* (*flaA*-SVR). Nas 18 amostras de frango analisadas, 14 foram positivas para a presença de *Campylobacter* spp., por pelo menos um dos métodos testados (ocorrência de 77,8%). A carga microbiana variou de $<1,0 \times 10^1$ a $2,2 \times 10^3$ UFC/g, com apenas uma amostra acima do limite estabelecido (10^3 UFC/g) no Regulamento (CE) n.º 2017/1495. Em quatro cozinhas, detetaram-se eventos de contaminação cruzada entre o frango cru e duas tábuas de corte, duas bancas e um pano de cozinha. Verificaram-se níveis muito elevados de resistência à ciprofloxacina (100%) e à tetraciclina (94,4%). Uma elevada taxa de resistência à eritromicina foi também observada neste estudo (40,3%), contrariamente ao relatado pela EFSA em 2016. Os isolados de *C. coli* apresentaram uma maior resistência do que os de *C. jejuni*, para todos os agentes antimicrobianos. Além disso, verificou-se que 63,9% dos isolados apresentaram multirresistências, dos quais 75,6% eram *C. coli*. A tipagem por PFGE mostrou uma elevada diversidade entre os isolados, assim como a tipagem de *flaA*-SVR (29 pulsotipos, 16 tipos de

alelo de *flaA* e 8 tipos de péptido de *flaA*). Estes resultados destacam a capacidade de disseminação de estirpes de *Campylobacter* resistentes no ambiente através da carne de frango, assim como a necessidade de educar o consumidor para um manuseio adequado dos produtos de carne de aves crua.

Palavras-chave: *Campylobacter* spp.; frango; contaminação cruzada; práticas do consumidor; campilobacteriose

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List of Abbreviations

AMC	Amoxicillin-Clavulanic Acid
AMP	Ampicillin
a_w	Water Activity
CASFM	Comité de l'antibiogramme de la Société Française de Microbiologie
CFA	CampyFood Agar
CFU/g	Colony Forming Units per gram
CIP	Ciprofloxacin
CmeABC	Efflux pump encoded by a three-gene operon (*cmeA*, *cmeB* and *cmeC*)
COI	Cost Of Illness
D	Simpson's index of diversity
DALYs	Disability-Adjusted Life-Years
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleoside triphosphate
DSMZ	German Collection of Microorganisms and Cell Cultures GmbH
E	Erythromycin
ECDC	European Centre for Disease Prevention and Control
EDTA	Ethylenediaminetetraacetic Acid
EFSA	European Food Safety Authority
ETP	Ertapenem
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
flaA	Flagellin A gene
flaA-SVR	Flagellin A - Short Variable Region
flaB	Flagellin B gene
FoodNet	Foodborne Diseases Active Surveillance Network

glyA	Serine Hydroxymethyltransferase gene
GN	Gentamicin
hipO	Hippuricase gene
ISO	International Organization for Standardization
KpnI	Restriction enzyme from *Klebsiella pneumoniae* OK8
mCCD agar	Modified Charcoal Cefoperazone Deoxycholate agar
MDR	Multidrug Resistant
MLST	Multilocus sequence typing
MOMP	Major Outer Membrane Porin
P1 to P15	Experimental Households
PCR	Polymerase Chain Reaction
PFGE	Pulse-Field Gel Electrophoresis
Pilots A to C	Pilot Households
RFLP	Restriction Fragment Length Polymorphism
rRNA	Ribosomal Ribonucleic Acid
SmaI	Restriction enzyme from *Serratia marcescens*
SPASS	Simpósio Nacional Promoção de uma Alimentação Saudável e Segura
TAE	Tris-acetate-EDTA Buffer
TBE	Tris-Borate EDTA Buffer
TE	Tetracycline
tetO	Tetracycline resistance gene class O
UK	United Kingdom
UPGMA	Unweighted Pair-Group Method using Arithmetic Averages
USA	United States of America
VNBC	Viable But Non-Culturable State
WHO	World Health Organization
WP	Work Package

WP1	Work Package 1
XbaI	Restriction enzyme from *Xanthomonas badrii*

1. Introduction

1.1. Epidemiology of human campylobacteriosis

Foodborne diseases are an important cause of morbidity and mortality, which affects significantly the socio-economic development worldwide (Kirk et al., 2015). Due to the consumption of animal products, some zoonoses can be included in the foodborne diseases group. By definition, zoonosis is any disease transmissible from animals to humans, directly or indirectly (Directive 2003/99/EC).

According to the World Health Organization (WHO), the most common cause of bacterial foodborne diarrhoeal disease worldwide are *Campylobacter* spp. (Kirk et al., 2015). In 2018, the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC) disclosed campylobacteriosis as the most reported zoonosis in the European Union (EU) during the previous year. It has been so since 2005, showing an increasing trend over the years, which stabilized during 2013-2017. Despite the high number of human campylobacteriosis cases in 2017 (246,158 cases with a notification rate of 64.8 per 100,000 population) the fatality rate of this zoonosis was low (0.04%). Information on the species was provided by all Member States for 54.1% of the cases, of which 84.4% were caused by *Campylobacter jejuni*, 9.2% by *Campylobacter coli*, 0.1% by *Campylobacter lari*, 0.1% by *Campylobacter fetus* and 0.1% by *Campylobacter upsaliensis* (EFSA & ECDC, 2018b).

The reporting of foodborne outbreaks of human campylobacteriosis is mandatory according to Directive 2003/99/EC of 17th November 2003. The purpose of this directive is to proper monitor zoonosis, zoonotic agents and related antimicrobial resistance, as well as to ensure that foodborne outbreaks receive adequate epidemiological investigation and to enable the collection of the necessary information to evaluate relevant sources and trends.

In the United States of America (USA), it is estimated that *Campylobacter* infections affect more than 1.3 million people every year. Foodborne Diseases Active Surveillance Network (FoodNet) reports an incidence of 14 diagnosed cases per 100,000 people every year, so it is believed that many cases go undiagnosed or unreported. More cases occur during summer than winter and the majority of them are not part of recognized outbreaks. Campylobacteriosis have been often associated with poultry, raw dairy products, untreated water and produce, but only 1 out of 5 *Campylobacter* infections are travel-associated (Centers for Disease Control and Prevention, 2017).

In Europe, EFSA and ECDC reported Czech Republic (230.0 cases per 100,000), Slovakia (127.8), Sweden (106.1) and Luxembourg (103.8) as the highest country-specific notification rates observed in 2017. The highest proportions of cases acquired within the country (> 94%) were reported in the Czech Republic, Hungary, Latvia, Malta, Poland, Portugal, Romania and Slovakia while the Nordic countries (Finland, Iceland, Norway, Denmark and Sweden) showed a high proportion of travel-associated cases. Between 2013 and 2017, there was a clear seasonality in the number of confirmed campylobacteriosis cases reported in the EU, with peaks in the summer months and a smaller peak in January (EFSA & ECDC, 2018b).

The largest *Campylobacter* foodborne outbreak in the EU in 2016 was reported by Sweden, involving more than 3,000 domestic cases after consumption of poultry meat (EFSA & ECDC, 2017). These cases were later linked to incorrect washing of transportation boxes from farms to the slaughterhouse in February 2017 (Whitworth, 2018). This outbreak lasted until June 2017, resulting in almost the double number of human cases acquired within the country compared with previous years (EFSA & ECDC, 2018b).

Several studies have estimated the burden of campylobacteriosis, expressed as disability-adjusted life-years (DALYs) and cost of illness (COI). Recent estimates range from 22,500 DALYs in the USA (Scallan et al., 2015), 18,222 in Australia (Gibney et al., 2014), 3,633 in The Netherlands (Mangen et al., 2015) to 1,568 in New Zealand (Lake et al., 2010). The number of years lost due to sequelae of the infection has been the major factor of DALYs for *Campylobacter* (Gibney et al., 2014; Scallan et al., 2015). Campylobacteriosis is also one of the most expensive foodborne diseases in Europe and Oceania (Gibney et al., 2014; Mangen et al., 2015). In New Zealand, COI estimates for campylobacteriosis between 2006 and 2007 represented an average value of 134,000,000$ and a cost per case of 600$. In The Netherlands the estimates refer to 2011 and disclose a cost per year of 76,100,000€ and a cost per case of 706€.

1.2. Campylobacteriosis in Portugal

The epidemiological characteristics of campylobacteriosis in Portugal show that *C. jejuni* is the main species infecting humans. Moreover, the observed prevalence of *C. coli* in Portugal was higher than that reported for other western countries (2009-2012: 14.8%). The age group with the highest risk was children between 1 and 16 years of age (61.5%), but a high infection rate was also observed in infants aged between 26 days and

11 months (25.2%) (Duarte et al., 2013). However, the national panorama is still unknown, since studies on the prevalence of this zoonosis are scarce and of a regional nature.

In 2017, Portugal reported 596 cases of human campylobacteriosis, showing a high proportion of cases acquired within the country (94%). This data represents a low notification rate, of 5.8 in 100,000 population in the EU (EFSA & ECDC, 2018b).

Even though the general use of antibiotics for growth promotion is forbidden in the EU (Regulation (EC) No. 1831/2003), antibiotic resistance is widely observed. Data from human isolates in Portugal showed that, between 2003 and 2007, fluoroquinolone resistance was already a reality. In a study from five Portuguese hospital laboratories, 80.5% (n= 123; n *C. jejuni* = 110; n *C. coli* = 13) of the isolates were resistant to ciprofloxacin (Vicente et al., 2008). Later on, Duarte et al. (2013) tested the antimicrobial susceptibility of 125 clinical isolates (*C. jejuni* = 78; n *C. coli* = 47) isolated between 2009 and 2012 and reported a high rate of resistance, mainly from *C. coli* strains. All strains were resistant to nalidixic acid, 92.8% were resistant to fluoroquinolones and 76% resistant to tetracycline/doxycycline. In addition, 87.2% of these strains were multidrug resistant (resistant to at least three unrelated antibiotics). Such high prevalence exposes the need for a close surveillance.

Data from EFSA and ECDC show that resistance levels differ considerably between the two most important *Campylobacter* species, *C. jejuni* and *C. coli*. Seventeen member states reported a very high proportion (54.6%) of *C. jejuni* human strains that were resistant to ciprofloxacin in 2016, with extremely high proportions observed in Portugal (94.0%). Similar conclusions were noted for tetracycline and erythromycin, whose overall resistance in the EU was 42.8% and 2.1%, respectively; and the second highest proportions of resistance between participating member states were observed in Portugal (82.0% and 6.6%, respectively). Meanwhile, *C. coli* strains showed significantly higher proportions of resistant isolates. The antimicrobial resistance observed in the EU for ciprofloxacin, tetracycline and erythromycin were 63.8%, 64.8% and 11.0%, respectively. On the other hand, Portugal observed 100% resistance to ciprofloxacin, 91.2% resistance to tetracycline and 50.0% resistance to erythromycin in *C. coli* strains isolated from humans. For gentamicin resistance, low percentage of resistant isolates was verified in Portugal (*C. jejuni* – 0.6%; *C. coli* – 0%), even though the proportion of resistant *C. coli* isolates in the EU was higher than the *C. jejuni* isolates (C. jejuni – 0.4%; C. coli – 1.7%) (EFSA & ECDC, 2018a).

1.3. *Campylobacter* historical perspective

It is believed that the early history of the genus *Campylobacter* reports to 1886, when Theodore Escherich described non-culturable spiral-shaped bacteria, which he found in the colon of children with an enteric disease called "cholera infantum" (Kirst, 1985), followed by McFadyean and Stockman (1913) that reported the isolation of a Vibrio-like organism from aborted ovine foetuses. These bacteria were widely known by veterinarians that along the years were able to report their presence in foetuses, blood and faeces from different animals (Debruyne et al., 2008; Doyle, 1944). The first well-documented foodborne outbreak of *Campylobacter* infection in humans was milk-related, causing diarrhoea to 355 prisoners from two adjacent state institutions, which took place in Illinois in the year of 1938 (Levy, 1946). Despite Sebald and Véron's proposal for *Campylobacter* genus in 1963, the scientific community continued to refer to these species as *Vibrio fetus* and *Vibrio bubulus* (On & Harrington, 2001). Only in 1973, the interest in these bacteria arose due to the study of Butzler et al. (1973), demonstrating a high prevalence of these spiral shaped rods in human diarrhoeal cases. Additionally, the understanding of its growth conditions and the discovery of successful isolation methods, such as selective supplements added to a basal medium (Skirrow, 1977), enabled the isolation of new *Campylobacter* species from different diseases and environments during the 1970's and the 1980's (Debruyne et al., 2008).

Today, twenty-six bacterial species and nine subspecies belong to the genus *Campylobacter*, which has experienced extensive changes over the years and yet some parts of the current genus taxonomy are still a matter of controversy (Debruyne et al., 2008; Kaakoush et al., 2015; On, 2001). However, the most important species from the current public health perspective are considered to *C. jejuni* and *C. coli*, since 98% of all the confirmed human cases of campylobacteriosis are related to one of these species (Gilliss et al., 2013).

1.4. General characteristics of *Campylobacter* spp.

Campylobacter are Gram-negative, spiral, curved, rod-shaped and non-spore-forming bacteria with one or two polar flagella and the typical movement of corkscrew of the family *Campylobacteriacae* (Man, 2011). These bacteria do not ferment nor oxidize carbohydrates due to the absence of 6-phophofructokinase, which is an enzyme that catalyses one of the key reactions of glycolysis (Kelly, 2005). Thus, these small cells (0.2-0.8 μm x 0.5-5 μm) obtain

their energy sources from amino acids or tricarboxylic acid cycle intermediates – chemoorganotrophs (Debruyne et al., 2008).

Most *Campylobacter* spp. have a microaerophilic nature, which optimal cultivation conditions are 5% oxygen, 10% carbon dioxide and 85% nitrogen, and have a respiratory type of metabolism. Under normal atmospheric oxygen tension, *Campylobacter* cells undergo a morphological change from spiral to coccoid (Boucher et al., 1994). However, several species (*C. curvus*, *C. gracilis*, *C. concisus*, *C. rectus*, *C. showae*, *C. mucosalis* and *C. hyointestinalis*) require a hydrogen-enriched atmosphere or formate as an electron donor for microaerobic growth. In addition, certain species grow under strict anaerobic conditions with fumarate or nitrate as final electron acceptor (Debruyne et al., 2008; Hoepers et al., 2016; Kaakoush et al., 2015) due to the genus' highly complex respiratory chain, with a great variety of electron donors and acceptors, which allows the cell to perform aerobic and anaerobic respiration (Kelly, 2005).

Most foodborne bacterial pathogens are considered relatively robust organisms, since they need to survive adverse conditions in the food industry (the use of preservatives/disinfectants, pasteurization, low water activity, high-pressure, radiation, etc) and food processing by the consumer (cooking) as well as the application of food preservation techniques (refrigeration, freezing, modified atmospheres). Through this point of view, *Campylobacter* spp. would be an unlikely foodborne pathogen (Park, 2002). The most common *Campylobacter* species, thermotolerant *Campylobacter* (*C. jejuni*, *C. coli*, *C. lari* and *C. upsaliensis*), which are able to grow between 37 ºC and 42 ºC but are incapable to do so at temperatures below 30 ºC, have uniquely fastidious growth requirements and show an unusual sensitivity to environmental stresses (Fitzgerald & Nachamkin, 2007; Moore et al., 2005). These species are also highly susceptible to a number of other environmental conditions and are less able to tolerate environmental stresses than other foodborne pathogens such as: oxygen >5%; desiccation – water activity (a_w) < 0.987; osmotic stress – [NaCl] ≥ 2%; pH <4.9; temperature – D value of one minute at 70ºC and susceptible to pasteurization (72 ºC/15 seconds) (Blaser et al., 1980; Doyle & Roman, 1982; Fernandez et al., 1985; Gill & Harris, 1982; Lee & Newell, 2006; Lori et al., 2007; Silva et al., 2011). Thus, *Campylobacter*'s ability to multiply outside of an animal host and in food during their processing and storage is reduced. Nevertheless, *C. jejuni* and *C. coli* are the current leading causes of foodborne human gastroenteritis (EFSA & ECDC, 2018b; Fitzgerald & Nachamkin, 2007; Moore et al., 2005).

The species *C. jejuni* and *C. coli*, are closely related and the differentiation between these two species is difficult. Biochemically, they only differ in the ability to hydrolyse hippurate, for which *C. jejuni* is positive (Debruyne et al., 2008). These two species are characterized by a fast motility that is mediated by polar flagella, crucial structures to the pathogenicity of these bacteria. This organelle is composed of a major flagellin (FlaA) and a minor flagellin (FlaB). FlaA is thought to be essential for colonization of animals and humans, although both proteins are probably needed for full motility (Nachamkin et al., 1993b). However, there is a more complex role to *Campylobacter*'s flagellin that includes adherence, invasion of host cells, protein secretion, autoagglutination and biofilm formation (Guerry, 2007).

Campylobacter jejuni shows the ability to colonize a diverse range of hosts, but there is little understanding of the molecular basis of this species virulence. This bacterium presents a mechanism of a fast adaptation to a new host, which is thought to be based on multiple highly mutable sites in the genome - contingency loci (Jerome et al., 2011). This genus also achieves genetic diversity through natural horizontal transfer of plasmid and chromosomal Deoxyribonucleic Acid (DNA). For example, *tetO* gene transfers between *C. jejuni* strains in chickens for tetracycline resistance without selective pressure. This recombination between strains happens *in vitro* and *in vivo* and allows further generation of genetic diversity (Avrain et al., 2004). It is believed that this natural transformation may play an important role in the plasticity of the genome and the dissemination of new mechanisms, such as the resistance to antibiotics (de Boer et al., 2002).

The colonization of the human intestinal epithelium by thermotolerant *Campylobacter* often causes an acute watery or bloody diarrhoeal illness, fever and abdominal pain. Campylobacteriosis is usually self-limited and can last from three days to one week after an incubation period of approximately 24 h to 72 h, depending on the infectious dose (Man, 2011). In severe cases, individuals may develop post infection complications such as reactive arthritis, haemolytic-uremic syndrome, pancreatitis, irritable bowel syndrome, Guillain-Barré syndrome or Miller Fischer syndrome. Fewer cases develop bacteremia, sepsis and death (Lastovicaan & Allos, 2008; Mangen et al., 2015). It is described by several authors that *C. coli* has been isolated from human blood, cerebrospinal fluid, faeces or intestinal tract, gallbladder, and retroperitoneal abscess. In addition to these sites, *C. jejuni* has also been isolated from a gastric biopsy, thoracic wall, peritoneal fluid and urine (Blaser et al., 1986; Man, 2011).

1.5. *Campylobacter* spp. in poultry

Nowadays, *Campylobacter* can still be isolated from different animals such as dogs, cats, sheep, cattle, pigs and some wild animals like birds (Andrzejewska et al., 2013; Gilpin et al., 2008; Horrocks et al., 2009; Sahin et al., 2008; Williams et al., 2016). Nevertheless, it is well established that the main source of human infection is the consumption or handling of contaminated food, especially poultry meat (EFSA & ECDC, 2017; Silva et al., 2011). Generally, the bacterium colonizes the cecum and colon of birds in high concentrations (10^6-10^8) and the chickens remain colonized until slaughter (Dhillon et al., 2006; Horrocks et al., 2009; Wilson et al., 2008). According to van Gerwe et al. (2009) mainly horizontal transmission happens between chickens, being one colonized bird able to infect 2.37 birds per day on average. So, it is estimated to take 21 days for the first chicken to become infected in a flock and one week, after colonization of the first bird, to increase the within-flock prevalence from one infected bird to 95% of infected chickens in a 20,000 broilers flock. In a farm scale, it takes two to four weeks to colonize the majority of chickens, after the first broiler is infected (van Gerwe et al., 2009).

It is controversial if *Campylobacter* is a commensal microorganism in chickens, which is thought not to cause any clinical symptoms, or if the bacterium can affect the birds' welfare (Food and Drug Administration, 2012; Dhillon et al., 2006; Hendrixson & DiRita, 2004; Humphrey et al., 2014). There is now increasing evidence that *Campylobacter* colonization of poultry harms the bird and affects its performance and growth. The physiology of *C. jejuni* and *C. coli* point to a long evolution and adaptation of these bacteria to the avian host, as can be verified by its suitability to the chicken's body temperature of 42 ºC. This temperature may allow thermophilic species to regulate gene expression based on specific growth requirements, favouring motility and energy regulation (Williams et al., 2013). Besides the chicken's colon, several studies have reported the ability of *Campylobacter* to colonize other organs, mainly the liver (Jennings et al., 2011; O'Leary et al., 2009; Van Deun et al., 2008). This invasive behaviour suggests that *Campylobacter* in chickens should be seen as either a pathogen or an opportunistic microorganism (Williams et al., 2014). Additionally, and according to Humphrey et al. (2014), *C. jejuni* cannot be considered a commensal bacterium, since colonization of chickens is associated with intestinal inflammation.

Although it is seen as a fastidious organism, the transmission of *Campylobacter* from environmental sources is considered the main route for chickens' colonization, once this genus is highly prevalent in the environment (Murphy et al., 2006). Possible sources and

vectors for infection are contaminated drinking water or feed, wild animals, rodents, flies, as well as contaminated equipment, vehicles and shipping boxes. These sources may even be closely linked with each other (Mendonça et al., 2016). However, the prevalence of infection depends on the season of the year, the chickens' diet, the age of the animal, the size and the type of flock and the geography. Summer months, free-range and organic farms, flocks with more than 15,000 and more than 25,000 birds are risk factors that increase significantly the prevalence of infection (EFSA & ECDC, 2011; Ellis-Iversen et al., 2009; Hendrixson & DiRita, 2004; Jorgensen et al., 2011; Nather et al., 2009).

The survival of *Campylobacter* in water is promoted by several factors like the microaerophilic environment provided by standing waters, biofilm formation, the transition to a viable but non-culturable state (VNBC) and its ability to invade and multiply within protozoan vectors like *Acanthamoeba* (Olofsson et al., 2013; Rollins & Colwell, 1986; Sparks, 2009). Once it is in the water, the microorganism can stay undetected and enter the food chain through the drinking water or washing of equipment/vehicles (Duffy & Dykes, 2009). Additionally, after a flock is colonized, the drinking water is often contaminated with the same strains of *Campylobacter* as the chickens and it is possible for more than one genotype to colonize a flock (Messens et al., 2009). In summer months, the presence of pests (flies and rodents) in farms is more common. However, by applying good agricultural practices during animal production such as, the placement of fly screens in aviaries or other pest control measures, it is possible to reduce the prevalence of *Campylobacter* infection in chickens (Bahrndorff et al., 2013). Persistent clones in a confined geographical area can also be responsible for successive infection in flocks (Kudirkienė et al., 2010), but improving hygienic measures as well as the health and welfare of the animals contributes to reducing broiler colonization (Bull et al., 2008).

An example of a simplified flow diagram of the processing of fresh poultry is presented in Figure 1, which includes further processed sub-products and service operations. Faecal contamination of chicken meat can occur at the slaughter processing steps of scalding, defeathering, evisceration, and washing (Nauta et al., 2009). During *C. jejuni* life cycle, the bacterium is exposed to highly variable oxygen concentrations. Therefore, it must be able to survive high environmental oxygen tensions, resist the oxidative stresses encountered in vivo and adapt to the severe oxygen limitation of the gut. Exposure to oxygen results in the inactivation of some oxygen sensitive enzymes and production of toxic reactive oxygen species, such as hydroxyl radical and hydrogen peroxide, which might lead to protein and nucleic acid injury. Despite *Campylobacter*'s sensitivity to atmospheric oxygen tension, it can

stay viable on chicken meat surfaces (Kelly, 2008). Several studies mention that some *Campylobacter* strains contain enzymes involved in oxidative stress defence (Atack & Kelly, 2009; Kelly, 2008; Krieg & Hoffman, 1986). However, another possible mechanism of dealing with high oxygen tension is thought to be metabolic commensalism with aerobic microorganisms found on food, like *Pseudomonas* spp. and *Escherichia coli* (Ghafir et al., 2008; Hilbert et al., 2010).

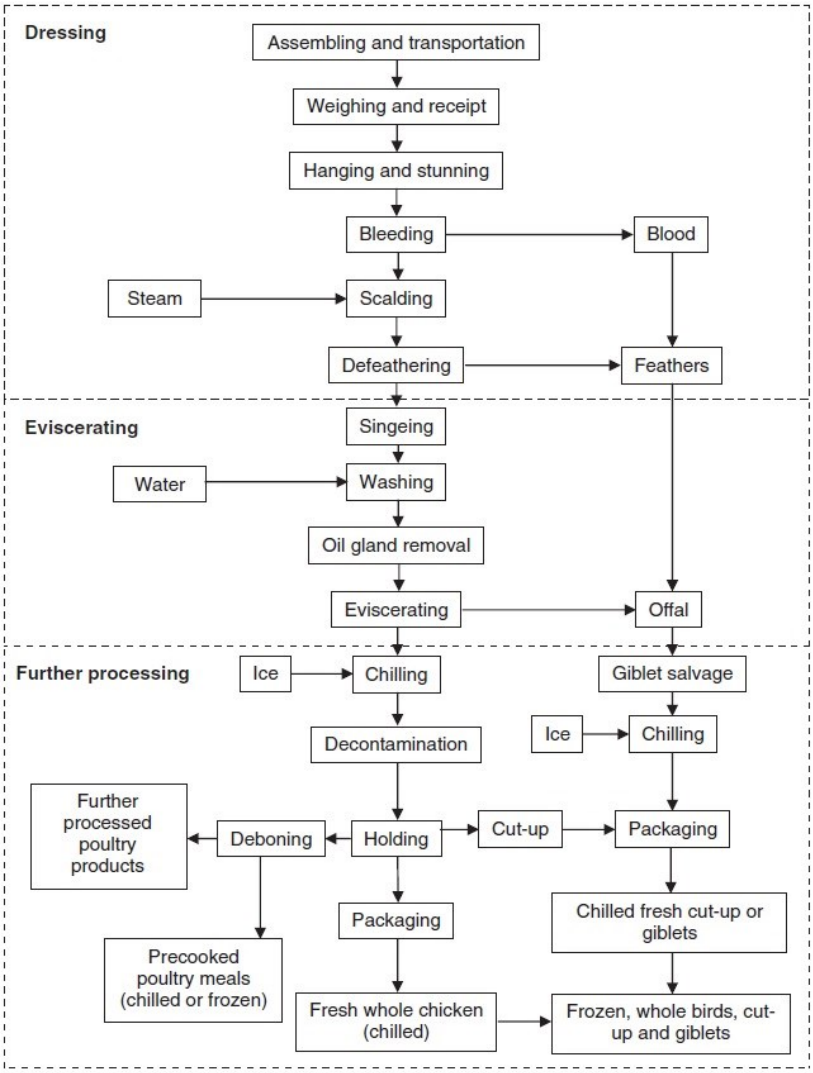


Figure 1 - Simplified flow diagram for processing fresh poultry *(in Chanona-Pérez et al., 2010)*

The control of campylobacteriosis involves a detailed understanding of its epidemiological aspects, which includes sources of contamination as well as the mechanisms and causes of its pathogenicity to humans (Wassenaar & Newell, 2000). Epidemiological studies of *Campylobacter* have resulted in, not only the implementation of hygienic and

biosecurity measures on rearing and slaughtering of poultry, but also diet altering, use of additives, pre- and probiotics at the farm level with the objective of reducing human exposure (Gellynck et al., 2008; Meunier et al., 2016; van de Giessen et al., 1998). These measures likely contributed to a reduction in the bacterial load of poultry. However, contaminated meat is still on the market and the epidemiology of *Campylobacter* in poultry is under-explored (Bull et al., 2008; Humphrey et al., 2014).

Despite all the effort, *Campylobacter* spp. is able to survive, as it is possible to find at European retail sale: 37.4% of broiler carcasses out of 13,445 tested positive for *Campylobacter* presence in 19 European countries. Not a very different result was reported for turkey meat: 31.5% of turkey carcasses out of 1,028 tested positive for *Campylobacter* spp. in eleven European countries (EFSA & ECDC, 2018b). In the United Kingdom (UK), broiler's neck samples from retail carcasses showed 71% of positive samples between December 2014 and February 2015 while one year later the percentage decreased to 63.5%. Poultry industry in the UK is adopting the trimming of the chicken's neck skins in order to remove the most contaminated part of the carcass and consequently, lower the bacterial load entering consumers' houses (Food Standards Agency, 2016).

According to EFSA, further reduction in the prevalence of *Campylobacter*-positive flocks is considered necessary. It is estimated that the chicken reservoir as a whole accounts for 50% to 80% of human cases of campylobacteriosis (EFSA, 2010b; EFSA & ECDC, 2018b). Therefore, a systematic approach should be considered, making the process hygiene criteria gradually stricter over time. Since January 1st 2018, a new process hygiene criterion is laid out in Regulation (EC) No 2017/1495, amending Regulation (EC) No 2073/2005 as regards *Campylobacter* in broiler carcases. This criterion presents a limit of <1,000 colony forming units (CFU)/g, that applies to poultry meat samples taken for official control, and aims at lowering contamination of carcases during the slaughtering process. In 2018, only two member states shared quantitative data, Spain and the UK reported 44% and 3.8% of carcasses with contamination levels above 1,000 CFU/g, respectively, reinforcing the need for official control (EFSA & ECDC, 2018b; Food Standards Agency, 2018).

1.6. Cross-contamination events

The high prevalence of *Campylobacter* in poultry meat associated with the high consumption of this type of meat makes this product a major vehicle for consumer's exposure to this bacterium. Once *Campylobacter* positive meat is brought into the kitchen, it can serve

as a source for cross-contamination to the hands of the food handler, other foodstuffs, utensils and surfaces during meal preparation (EFSA, 2010b). Although the number of cells transferred depends on the number of the bacteria on the poultry (Verhoeff-Bakkenes et al., 2008), it is estimated that handling, preparation and consumption of broiler meat accounts for 20% to 30% of human cases of campylobacteriosis (EFSA, 2010b).

Infection happens through cross-contamination to ready-to-eat or cooked products, direct hand-to-mouth transfer during food preparation as well as from the consumption of undercooked poultry meat (EFSA, 2010b). The infectious dose required for *Campylobacter* infection is thought to be low (500 cells), but acute illness requires a much higher dose. This means that a single drop of juice from raw chicken can have enough *Campylobacter* cells to infect a person (Food and Drug Administration, 2012; Centers for Disease Control and Prevention, 2017).

Few studies have been performed to evaluate the transfer of *Campylobacter* during handling and preparation of naturally contaminated poultry in consumers' houses. At retail level, a study in the UK reported outer packaging of chicken meat positive for *Campylobacter* contamination between July 2014 - February 2015 (7.5%) and in the same period of 2016 (5.6%) (Food Standards Agency, 2016). Cross-contamination events in the household were also studied in the UK, where 20 consumers were asked to prepare a meal with naturally contaminated chicken after rinsing and portioning it on a cutting board. After food preparation, cutting boards (25%), hands (15%), surroundings (10%), kitchen cabinets (5%), kitchen doors (5%) and oven handlers (5%) tested positive for the presence of *Campylobacter* (Cogan et al., 1999). In order to mimic what happens in home kitchens, Guyard-Nicodème et al. (2013) studied the transfer of *Campylobacter* between naturally contaminated raw chicken legs, the cutting board and a cooked chicken slice. This laboratory study showed that the transfer from the cutting board to the cooked chicken happened in 28.9% of the cases, after the plastic board contacted 7 minutes with each of the samples separately (Guyard-Nicodème et al., 2013).

Since *Campylobacter* can transfer and attach to surfaces, cross-contamination needs to be avoided. Washing the poultry causes contamination of the surroundings, so transferring the poultry from the packaging directly to the oven/pot should be done instead. After handling poultry meat, cleaning may not be as effective as consumers think. The use of hypochlorite disinfectant in addition to detergent and hot water results in a significant decrease in the number of positive sites compared to those found to be contaminated after washing only with detergent and water alone (Cogan et al., 1999).

The reduction of consumers' risks is possible through prevention of cross-contamination events at home kitchens, hand washing during food preparation as well as heating food products at temperatures high enough to kill microorganisms. Yet, this requires increased consumer awareness. In a 2014 survey by the Food Standards Agency (UK), levels of awareness of *Campylobacter* were proven to be below that of other forms of food poisoning. Only 28% of the people had heard of *Campylobacter*, compared with 90% who had heard of *Escherichia coli* and *Salmonella* (EFSA, 2014; Skarp et al., 2016).

2. Aims and outline of the thesis

This work was developed in the scope of SafeConsume project (SafeConsume, 2017), funded by Horizon2020, that aims to create new strategies to help the consumer to mitigate food risks by increasing knowledge and skills in responsible and safe food handling and consumption, and ultimately to reduce the number of foodborne illness at consumer stage. The project started in April 2017 and will run for five years, with 32 partners in 14 countries in Europe, and focuses on the five most significant foodborne hazards in Europe: *Campylobacter* spp., *Listeria monocytogenes*, norovirus, *Salmonella* spp. and *Toxoplasma gondii*.

The work presented in this dissertation forms a part of investigations carried under the first Work package (WP) of SafeConsume (WP1- Characterization of consumer behaviours and barriers). In this WP, consumers' food handling practices, possibilities and barriers to food safety in selected households, including consumers with different vulnerabilities and levels of awareness to food safety, were evaluated. A total of 75 households, covering five European countries, were visited for observation and interview during purchase at retail, transportation, storage, food preparation and serving.

The overall objective of this research was to evaluate possible cross-contamination events that can contribute to the spread of *Campylobacter* spp. in domestic kitchen environments during food preparation, as well as characterize pheno- and genotypically the recovered isolates. Eighteen individual Portuguese households were included in the study, and recruited consumers were asked to prepare a recipe that included poultry and a raw vegetable salad. Observations of raw poultry handling were carried out in the volunteers' private kitchens, and food and surface samples were collected for microbial analysis. *Campylobacter* spp. isolates recovered from food and surfaces positive samples were collected and further characterized by phenotypic and genotypic methods, including: species identification, susceptibility to antibiotics, and molecular subtyping by pulse-field gel electrophoresis (PFGE) using *SmaI* and *KpnI* enzymes and by sequencing of the Short Variable Region (SVR) of *flaA* flagellin gene in order to identify possible cross-contamination routes via hands, cutlery, cutting board, etc. A graphical representation of the outline of the thesis is shown in Figure 2.

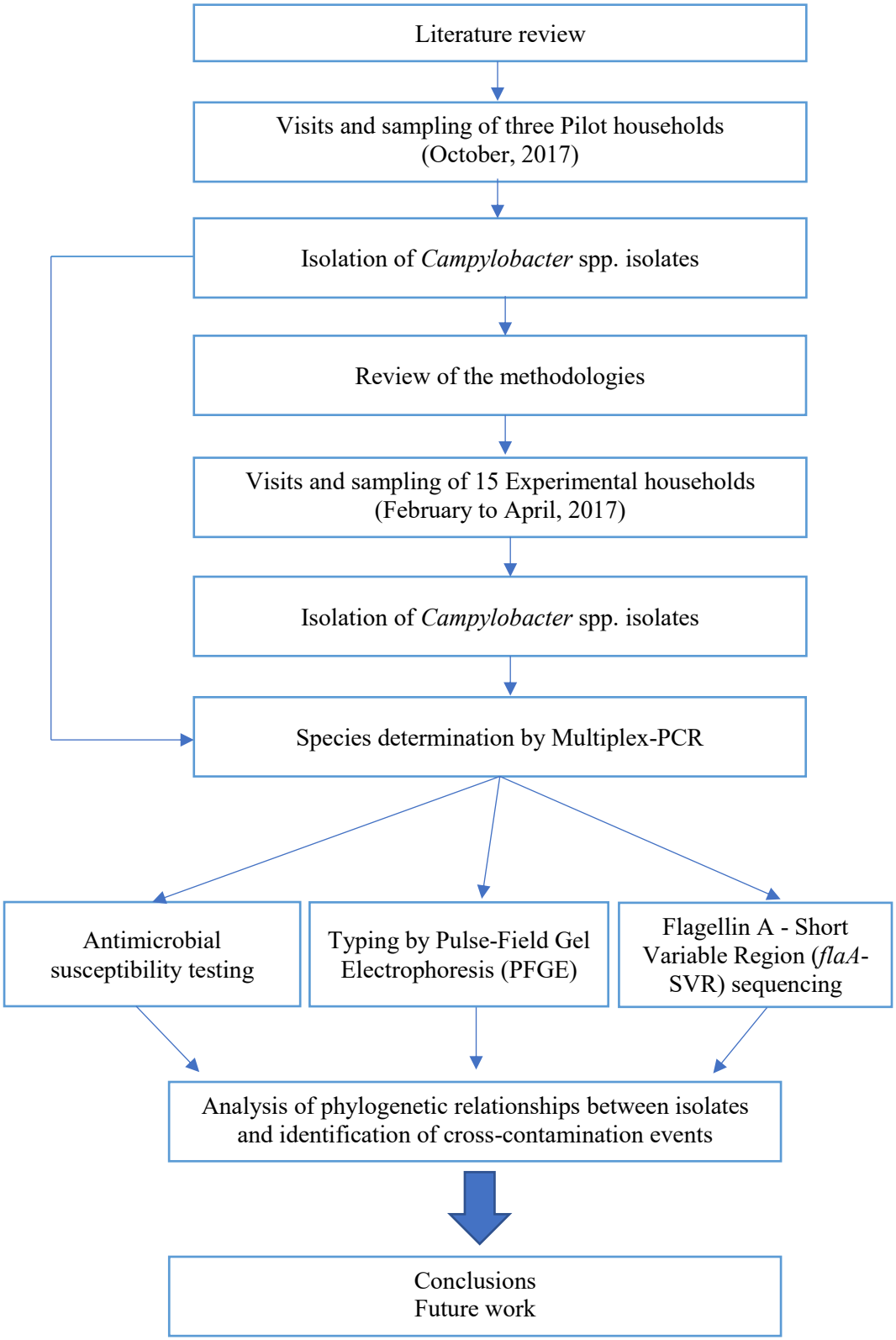


Figure 2 - Graphical representation of the outline of the thesis.

Some of the work presented in this thesis was displayed in one national conference:

- 5º Simpósio Nacional "Promoção de uma Alimentação Saudável e Segura – SPASS 2018", Instituto Nacional de Saúde Doutor Ricardo Jorge, Lisbon, Portugal, September 27th, 2018

This work was also accepted for a poster presentation in one international conference:

- IAFP's European Symposium on Food Safety, Nantes, France, April 24-26th 2019

Additionally, one paper is in preparation to be submitted for publication in a peer-reviewed scientific journal.

3. Materials and Methods

3.1. Household recruiting and transdisciplinary fieldwork

In the beginning of the fieldwork study (October 2017), with the aim to establish standard protocols for microbial sampling and analysis, as well as the methodological principles, tools and procedures applied in WP1, three pilot studies (Pilot A, Pilot B, and Pilot C) were conducted in each one of the six WP-partner countries (Portugal, France, Norway, Hungary, Romania, and UK). For this initial test, the three participants were recruited by word of mouth from the researchers' circle of friends and family members. Subsequently, after discussions among partners, refinement and modification of protocols and observational templates, 15 additional households (P1 to P15) were recruited for the final study, between February and April 2018, through a subcontracted professional recruitment agency. Households were selected based on recruitment criteria to include specific demographic groups identified as either vulnerable (elderly, young children and pregnant women) or of high risk (young men). Participants in the study were informed about the objectives of the study and procedures involved, and were required to sign the consent form before they were formally enrolled in the study. Detailed information on pilot and experimental households are presented in Table 1.

The interdisciplinary field study, carried out by a team of two microbiologists and one social-scientist, included a visit of approximately 4 hours to each participant household, focused on four specific stages: consumers' shopping routine; transportation of food between supermarket and home; food storage routine; and food preparation. In this study, the focus was centred on the experimental settings and results of "food preparation" stage.

Table 1 - Detailed information on pilot and experimental households investigated in this study

Code	Sampling date	Target group	Income	Location
Pilot A	02/10/2017	Elderly	n/a	Urban
Pilot B	03/10/2017	Single man (<30 year old)	n/a	Urban
Pilot C	03/10/2017	Family	n/a	Urban
P01	19/02/2018	Pregnant/Family	Medium	Urban
P02	20/02/2018	Pregnant/Family	Medium	Rural
P03	21/02/2018	Elderly	High	Urban
P04	26/02/2018	Elderly	Medium	Urban
P05	27/02/2018	Family	Medium	Urban
P06	28/02/2018	Elderly	Medium	Urban
P07	05/03/2018	Elderly	Medium	Urban
P08	06/03/2018	Family	Medium	Urban
P09	07/03/2018	Single man (<30 year old)	High	Urban
P10	19/03/2018	Elderly	Low	Urban
P11	20/03/2018	Family	Medium	Urban
P12	21/03/2018	Single man (<30 year old)	Medium	Urban
P13	03/04/2018	Single man (<30 year old)	Medium	Urban
P14	05/04/2018	Elderly	Low	Urban
P15	05/04/2018	Pregnant/Family	Medium	Urban

n/a – not available

3.2. Observation of food preparation sessions in domestic kitchens and sampling procedures

The food preparation sessions were carried out in the home kitchens of the participants and recorded with a digital video camera (used to capture food handling practices of the subjects) and a voice recorder. The participants were asked to prepare a recipe with poultry and a raw vegetable salad of their own choice, the way they would normally do, and were encouraged to always describe what they were doing/thinking/considering throughout the session. At the end, the participants were instructed to leave the kitchen as they would normally leave it after food preparation, using the habitual cleaning agents and procedures. The volunteers were aware that their actions and speech were being monitored, but not ware that their household food safety practices were the subject of analysis. At specific time points microbiological samples of the food, surfaces and utensils were collected and tested for the presence and/or enumeration of *Campylobacter* as further detailed.

In every food preparation session the domestic kitchen was sampled immediately before the participants had started the food preparation and after their normal cleaning procedures. Samples were taken from pre-determined sites, including: tap handle; cabinet, drawer and refrigerator handles; and the counter top surface. Other surfaces were sampled

depending on observed behaviours during the individual food preparation sessions, for example: cutting boards (before and after food preparation if used by the participant, and after cleaning procedures but only if hand-washed) and the sink (if the poultry was washed before preparation). At the end of the sessions, the kitchen's cloth and/or sponge (if used), and hand towel (if touched with poorly cleaned hands after handling raw chicken) were also collected and placed into sterile plastic bags. In the three pilot households, sampling sites were swabbed with a sterile cotton tipped swabs, pre-moistened in a sterile isotonic salt solution (Ringer solution, Biokar Diagnostics, Solabia Group, Pantin, France) using sterile techniques; the swabs were subsequently place in sterile 13 ml plastic tubes. In experimental households, sampling sites were swabbed with sterile swabbing cloths (SodiBox, Nevez, France); upon completion, the cloths were carefully placed back in their original plastic bag. Food samples collected for analysis included raw poultry parts and raw vegetable salad, chosen by the participants and placed inside sterile plastic bags upon our request. Food and surface swab samples were kept in coolers in the field while sampling was being completed, then immediately taken to the laboratory and stored at 4 ºC until microbial testing (within 18h).

3.3. *Campylobacter* spp. detection and enumeration

Campylobacter detection was performed according to International Organization for Standardization (ISO) 10272-1:2017. Food samples were aseptically weighed into sterile stomacher bags and sterile Bolton broth (VWR Chemicals, Leuven, Belgium) with 5% defibrinated horse blood (Thermo Fisher Scientific, Massachusetts, USA) was added in the proportion of x to $9x$ (minimum 10 g). After homogenization for 1 minute in a stomacher (Interscience, Saint Nom, France), the samples were incubated for 48 h at 41.5 ºC under microaerophilic conditions.

Sampling site swabs from the pilot households were homogenised with 10 ml of sterile Bolton broth with 5% defibrinated horse blood in the stomacher for 1 minute.

Swabbing cloths from the experimental households and kitchen cloths were homogenised with 25 ml of sterile buffered peptone water (BPW, Bio-Rad Laboratories, California, USA) in a stomacher for 1 minute, while sponges were homogenised with 50 ml of BPW, due to the foaming during homogenization, and hand towels were homogenized with 225 ml of BPW. Afterwards, a 1 ml aliquot of the homogenate was inoculated into 9 ml Bolton broth tube with 5% defibrinated horse blood, and incubated at 41.5 ºC under microaerophilic conditions for 48h. This methodology was chosen so the same initial sample

could be tested for the presence of *Campylobacter* spp., *Salmonella* spp. and/or *L. monocytogenes* as well as the enumeration of mesophilic bacteria and *Listeria* spp. (parameters not investigated in the scope of this work).

The selective solid medium chosen for inoculation of the enrichment culture were Modified Charcoal Cefoperazone Deoxycholate agar (mCCD agar, VWR Chemicals) and CampyFood Agar (BioMérieux, Marcy-l'Étoile, France). Both were incubated for 48 h at 41.5 ºC in a microaerobic atmosphere. Then, up to five typical colonies of each plate were sub-cultured in Columbia agar (Merck Millipore, Massachusetts, United States) with 5% defibrinated horse blood and incubated under the same conditions for 24h for further confirmation.

3.4 Enumeration technique

Enumeration of *Campylobacter* spp. was performed only in poultry samples according to ISO 10272-2:2017. Twenty-five grams of poultry were added to 225 ml of sterile BPW, homogenized in a stomacher for 1 minute, and enumeration was performed by spread plate count of 1 ml and 0.1 ml of buffered peptone water in mCCD agar plates. Plates were incubated under microaerobic atmosphere at 41.5 °C for 48 h, before typical *Campylobacter* colonies were counted. Up to five typical colonies of each plate were then sub-cultured in Columbia agar under the same conditions for 24 h for further confirmation tests.

3.5. Identification of presumptive colonies of *Campylobacter* spp.

Presumptive *Campylobacter* spp. isolates were subcultured in mCCD agar plates and confirmed by standard procedures, including observation of haemolysis after 24h incubation, microscopy of a freshly prepared bacterial suspension and oxidase test.

To differentiate bacteria based on their haemolytic properties, *Campylobacter* presumptive colonies were cultured on blood agar for 24 h. Haemolysis is the disruption of red blood cells and release of their haemoglobin. In β-haemolysis, a clear zone around the colonies is formed due to the total destruction of red blood cells. In α-haemolysis, bacteria produce a greenish zone of incomplete cell destruction around the colony, as a consequence of haemoglobin oxidation. Finally, in γ-haemolysis, the agar under and around the colony is unchanged (Tille, 2014; Willey et al., 2014). Although the most common species of

Campylobacter are not considered to be haemolytic on blood agar (Smibert, 1984), some studies report the existence of certain species of *Campylobacter* that express haemolytic properties after 4 days of incubation on blood agar (Arimi et al., 1990). Therefore, in this study only γ-haemolytic colonies with an incubation time of 24 h were retained for further examination.

Oxidase test was performed according to ISO 10272-1 (2017) and Shields & Cathcart (2010) through the filter paper test method. Using a sterile toothpick, a well-isolated colony from the blood agar plate was streak onto the moistened filter paper with the oxidase reagent (Merck Millipore, Massachusetts, United States). Oxidase-positive colonies were retained for further examination.

The microscopy of a freshly grown colony of the blood agar plate intended to examine the morphology and motility of the cells. *Campylobacter* cells are small curved bacilli with a characteristic corkscrew darting ISO 10272-1 (2017). Isolates with both characteristics were retained for Multiplex Polymerase Chain Reaction (PCR).

Isolates that fulfilled the criterion were further stored at -80 ºC in defibrinated horse blood with 15% (v/v) glycerol (VWR Chemicals) and emulsified by vortexing, as described by Gorman & Adley (2004).

3.6. Multiplex Polymerase Chain Reaction (PCR)

3.6.1. DNA extraction

Isolates were grown at 41.5 ºC in Columbia agar under microaerophilic conditions for up to 48 h. Cells were suspended in an isotonic solution (Ringer solution) to an optical density range of 0.57-0.82 (610 nm wavelength). Commercial DNA extraction kit (GRS Genomic DNA Kit – Bacteria, GRiSP Research Solutions, Porto, Portugal) was used along with Gram-negative bacteria protocol during this work. All DNA samples were stored under -20 ºC until further use.

3.6.2. Multiplex PCR conditions

The multiplex PCR assay was performed to simultaneously detect genes from the three major clinically relevant *Campylobacter* spp.. In this study, the optimization of the method

described by Wang et al. (2002) was performed for the identification of 23S ribossomal RNA (rRNA) from *Campylobacter* spp., the *hipO* gene (hipuricase) from *C. jejuni* and the *glyA* gene (serine hydroxymethyltransferase) from *C. coli* and *C. lari*. PCR conditions used were a 30-cycle reaction with 6 minutes initial denaturation at 95 ºC, 30 seconds denaturation at 95 ºC, 30 seconds annealing at 52 ºC, 30 seconds extension at 72 ºC and 7 minute final extension at 72 ºC using a 25 µl reaction mixture.

Table 2 - Oligonucleotide sequences of the primers used in the multiplex PCR assay, target genes and expected sizes for the amplified products.

Target gene	Primer *	Product size (bp)	Sequence (5'-3')
23S rRNA	23SF	650	TATACCGGTAAGGAGTGCTGGAG
	23FR		ATCAATTAACCTTCGAGCACCG
C. jejuni hipO	CJF	323	ACTTCTTTATTGCTTGCTGC
	CJR		GCCACAACAAGTAAAGAAGC
C. coli glyA	CCF	126	GTAAAACCAAAGCTTATCGTG
	CCR		TCCAGCAATGTGTGCAATG
C. lari glyA	CLF	251	TAGAGAGATAGCAAAAGAGA
	CLR		TACACATAATAATCCCACCC

* Primers supplied by Stab Vida (Caparica, Portugal)

Each PCR tube contained 1x Taq Buffer with KCl (100 mM Tris-HCl, pH 8.8, and 500 mM KCl) (Thermo Fisher Scientific, Massachusetts, USA), 2 mM of $MgCl_2$ (Thermo Fisher Scientific), 200 mM of deoxynucleoside triphosphate (dNPTs) mixture (Thermo Fisher Scientific) and 1.0 U of Taq DNA polymerase (Thermo Fisher Scientific). *Campylobacter* specific primers were also added to each tube: 0.2 µM of 23S rRNA primer, 1 µM of *C. coli* primer, 0.5 µM of *C. jejuni* primer and 0.5 µM of *C. lari* primer. The DNA template added consisted of 1µl and the volume was adjusted to 25 µl with sterile ultrapure water.

DNA amplification was carried out in a T100 thermal cycler (Bio-Rad Laboratories) and PCR products were separated by electrophoresis on a 1.5% (w/v) agarose gel (GRS Agarose LE, GRiSP Research Solutions) with Midori Green (Nippon Genetics Europe GmbH, Dueren, Germany) in 1x Tris-acetate-Ethylenediaminetetraacetic (EDTA) buffer (TAE) (Merck Millipore) at 80 V for 45 minutes. Reference strains used as controls for the PCR assays were German Collection of Microorganisms and Cell Cultures GmbH (DSMZ) 4688 (*C. jejuni*), DSMZ 4689 (*C. coli*) and DSMZ 11375 (*C. lari*).

3.7. Antimicrobial susceptibility testing

The antimicrobial susceptibility test was performed using the disk diffusion method according to Comité de l'antibiogramme de la Société Française de Microbiologie (CASFM) and European Society of Clinical Microbiology and Infectious Diseases (EUCAST) (CASFM & EUCAST, 2017). Thirty-one *C. jejuni* and 41 *C. coli* were cultured in mCCD agar (VWR Chemicals), suspended in an isotonic solution (Ringer solution), adjusted to match the 0.5 McFarland turbidity standard and cultured in Mueller-Hinton Agar with 5% defibrinated horse blood and 20 mg/L β-Nicotinamide adenine dinucleotide (β-NAD, Sigma-Aldrich, St. Louis, Missouri, USA). Antimicrobial susceptibility was performed using standard discs (Oxoid, Hampshire, England) containing ciprofloxacin (5 µg), tetracycline (30 µg), gentamicin (10 µg), erythromycin (15 µg), ampicillin (10 µg), amoxicillin-clavulanic acid (20/10 µg) and ertapenem (10 µg). CASFM & EUCAST breakpoints for *Campylobacter* spp. were used to assess resistance. The plates were incubated under microaerophilic conditions (GENbox, BioMérieux) at 37 ºC for 48 h. The reference strain used as control was DSMZ 4688 (*C. jejuni*), as recommended by CASFM & EUSCAST (2017).

3.8. Subtyping by Pulse-Field Gel Electrophoresis (PFGE)

Campylobacter spp. isolates were cultured on Columbia blood agar (COS, BioMérieux) under microaerophilic conditions at 41.5 ºC for 24 h. Molecular subtyping of the isolates was performed according to the PulseNet protocol (PNL03 last update July 2017 - https://www.cdc.gov/pulsenet/pdf/campylobacter-pfge-protocol-508c.pdf). Reference strains used as controls were DSMZ 4688 (*C. jejuni*) and DSMZ 4689 (*C. coli*). The selected restriction enzymes for all *Campylobacter* isolates were *Sma*I and *Kpn*I (Thermo Fisher Scientific). *Salmonella* Braenderup plugs restricted with *Xba*I were used as the molecular size standard.

Restricted plugs were loaded into a 1% SeaKem Gold agarose gel (Lonza Group AG, Basel, Switzerland) and electrophoresed in 0.5x Tris-Borate EDTA Buffer (TBE) (GRiSP Research Solutions), at 6 V/cm and an included angle of 120º on a Chef DR III system (Bio-Rad Laboratories). *Sma*I gel's run time was 19 h while *Kpn*I gel's run lasted for 18 h. The electrophoresis conditions used were the same mentioned on the PulseNet protocol. Gels were stained using ethidium bromide solution (MP biomedicals, Santa Ana, California, USA) and photographed using Gel Doc XR+ System with Image Lab Software (Bio-Rad

Laboratories). BioNumerics v.7.6.2 (Applied Maths, Sint-Martens-Latem, Belgium) was used for numerical analysis of the enzymes restriction patterns and Dice coefficient was used for similarity analysis (position tolerance of 1.5%). PFGE patterns were clustered using the Dice coefficient and the unweighted pair-group method using arithmetic averages (UPGMA). Classification of isolates into different *Sma*I and *Kpn*I patterns was visually validated, and a similarity threshold of ≥ 98% was used to define isolates belonging to the same PFGE types, that were further designated by numbers (e.g., 001).

3.9. *Flagellin A* - Short Variable Region *(flaA*-SVR) sequencing

The DNA used for the PCR assay was obtained as previously described in section 3.6.1. The *flaA*-SVR was amplified using primers FLA242FU: 5'-CTATGGATGAGCAATTWAAAAT-3' and FLA625RU: 5'-CAAGWCCTGTTCCWACTGAAG-3' (Meinersmann et al., 1997). All reactions contained 1 µl of DNA template, 12.5 µl KAPA HiFi HotStart ReadyMix PCR Kit (0.3 mM of each dNTP, 2.5 mM $MgCl_2$ and 0.5 U of KAPA HiFi HotStart DNA Polymerase, Kapa Biosystems, Massachusetts, USA), *flaA*-SVR primers (Stab Vida) at a final concentration of 0.3 µM and sterile ultrapure water to a final volume of 25 µl, as recommended by the manufacturer.

DNA amplification was carried out in a T100 thermal cycler (Bio-Rad Laboratories). A 30-cycle reaction was used with 3 minutes initial denaturation at 95 ºC, 20 seconds denaturation at 98 ºC, 15 seconds annealing at 60 ºC, 15 seconds extension at 72 ºC and 1 minute final extension at 72 ºC, after optimization of the protocol mentioned in Nachamkin et al. (1993a). Reference strains used as controls for the PCR assays were DSMZ 4688 (*C. jejuni*) and DSMZ 4689 (*C. coli*). The desired 321 base-pair band was detected by electrophoresis on a 1.5% (w/v) agarose gel (GRS Agarose LE, GRiSP Research Solutions) at 80 V for 45 minutes. PCR products were then purified with a GRS PCR & Gel Band Purification Kit (GRiSP Research Solutions).

Sequence data were generated using FLA242FU and FLA625RU primers through GATC Biotech (Konstanz, Germany) and Macrogen (Seoul, South Korea) services. Data was analysed with Molecular Evolutionary Genetics Analysis – Mega X Software version 10.0.5 (Kumar et al., 2018) and aligned using ClustalW algorithm (Thompson et al., 1994). Aligned sequences were analysed in PubMLST database (https://pubmlst.org/campylobacter/) (Jolley et al., 2018).

3.10. Simpson's Index of Diversity

The discriminatory ability of the typing methods used for the differentiation of isolates was calculated through the application of the Simpson's index of diversity (D) (Hunter & Gaston, 1988). This index describes the ability of a technique to type differently two unrelated strains sampled randomly and taken from the population of a given species (van Belkum et al., 2007). According to Hunter & Gaston (1988), values range between 0 and 1, being an index greater than 0.90 considered desirable if the typing results are to be interpreted with confidence.

The Simpson's index of diversity with 95% confidence interval was determined for each method and each species (*C. jejuni* or *C. coli*) using the online tool for quantitative assessment of classification agreement (http://www.comparingpartitions.info/) and confirmed by calculation through the given formula:

$$D = 1 - \frac{\sum_{j=1}^{S} n_j \left(n_j - 1\right)}{N(N-1)}$$

Where N is the total number of strains in the sample population, S is the total number of types described, and n_j is the number of strains belonging to the j^{th} type.

4. Results and Discussion

During the present study, three pilot households were visited in October 2017 and another 15 experimental households between February and April 2018. Different chicken, surface samples, sponges, cloths and hand towels were collected throughout food preparation and microbial analysis were performed. In this chapter, detection and enumeration results are discussed separately for chicken and surface samples, but for the characterization of isolates no separation was applied.

4.1. Occurrence of *Campylobacter* spp. in poultry meat

Table 3 summarizes the results obtained for the detection and enumeration of *Campylobacter*. In 18 chicken samples that were analysed during this work, four were negative (Pilot B, P05, P06 and P11), whereas 14 were positive for the presence of *Campylobacter* spp., at least by one of the tested methods. This represents an occurrence of 77.8%. The microbial load ranged from $< 1.0 \times 10^1$ to 2.2×10^3 CFU/g.

Ghafir et al. (2007) revealed a slightly lower *Campylobacter* occurrence in 25 g of chicken meat from Belgium slaughterhouses, production plants and retail level in 1997 and 1998 (71.0% and 72.6%, respectively). However, from 2000 to 2003 there was a decrease in the occurrence of this pathogen in chicken meat that ranged from 39.4% to 54.5%, due to the implementation of good hygiene practices during meat processing (Ghafir et al., 2007). Narvaez-Bravo et al. (2017) reported an even lower occurrence in chicken (23.5%) and turkey meat (14.2%) in retail meat samples purchased in Alberta, Canada. The results shown in the present study do not differ significantly from those reported in the latest EFSA (2010a) scientific report on *Campylobacter* prevalence in broilers. In 2008, Portugal evidenced a prevalence of 70.2% of contaminated poultry carcasses, with 24.5% of the meat samples hosting counts between 10^2 and 10^3 CFU/g, 12.1% between 10 and 10^2 CFU/g and 39% below 10 CFU/g. Comparing to other countries, Portugal was the 13th member state with the highest prevalence, but below EU average (75.8%) (EFSA, 2010a). In the same report, Spain presented a prevalence of 92.6% of contaminated poultry meat, but with higher microbial load – enumeration results above 10^3 UFC/g in 44.2% of the samples (EFSA, 2010a). However, a recent study reports a lower occurrence in Spanish chicken products – 39.4%, of which unpacked products were more contaminated than packed ones (García-Sánchez et al., 2018).

Table 3 - Detection and enumeration of *Campylobacter* spp. results in poultry samples collected from 18 Portuguese households.

House	Shopping place	Type of meat	Detection (in 10 g)	Enumeration (CFU/g)
Pilot A	Supermarket chain A	Free range chicken (package)	Positive	2.2×10^3
Pilot B	Supermarket chain B	Chicken breast (package)	Negative	$< 1.0 \times 10^1$
Pilot C	Supermarket chain C	Chicken breast steaks (package)	Positive	Present but $< 4.0 \times 10^1$
P01	Supermarket chain C	Chicken breast steak (package)	Positive	$< 1.0 \times 10^1$
P02	Supermarket chain A (butcher)	Chicken breast steaks cut into small pieces	Positive	1.6×10^2
P03	Supermarket chain A	Free range chicken thighs (package)	Negative	1.5×10^2
P04	Supermarket chain B	Chicken thighs (packaged)	Positive	$< 1.0 \times 10^1$
P05	Supermarket chain B	Chicken breast (package)	Negative	$< 1.0 \times 10^1$
P06	Supermarket chain C (butcher)	Whole chicken without skin	Negative	$< 1.0 \times 10^1$
P07	Supermarket chain C (butcher)	Whole free-range chicken	Negative	Estimated No. 9.0×10^1
P08	Supermarket chain D (butcher)	Chicken breast	Positive	Present but $< 4.0 \times 10^1$
P09	Supermarket chain B	Chicken legs (package)	Negative	Present but $< 4.0 \times 10^1$
P10	Street butcher shop	Whole chicken	Negative	1.4×10^2
P11	Supermarket chain A (butcher)	Whole chicken cut into pieces and without skin	Negative	$< 1.0 \times 10^1$
P12	Supermarket chain A (butcher)	Whole chicken	Negative	Present but $< 4.0 \times 10^1$
P13	Supermarket chain E	Chicken thighs (package)	Negative	4.1×10^2
P14	Supermarket chain A (butcher)	Whole chicken cut into pieces	Positive	Present but $< 4.0 \times 10^1$
P15	Supermarket chain C (butcher)	Whole chicken cut into pieces	Positive	Present but $< 4.0 \times 10^1$

According to Regulation (EC) No 2017/1495, in 2018 and 2019 the process hygiene criterion for broiler meat will be based on a limit of 10^3 CFU/g of *Campylobacter* spp. in 20 out of 50 carcass samples with neck skin after chilling. This number of samples will decrease to 15 out of 50 between 2020 and 2024 and 10 out of 50 starting from 2025 (Regulation (EC) No. 2017/1495). To the moment of this work, only one of the 18 analysed samples presented results above the established limit. Roccato et al. (2018) observed post

chilling enumeration results ranging from 3.09 to 3.32 Log CFU/g in samples from three slaughterhouses in Italy, demonstrating that much work remains to be done.

One hundred and forty one isolates were collected from detection and enumeration techniques, and initially identified as presumptive *Campylobacter* spp. according to microscopy examination, oxidase test, and haemolytic properties. Only 60 were further confirmed to belong to the *Campylobacter* genus (by a Multiplex-PCR assay), specifically 22 *C. jejuni* and 38 *C. coli*. Isolation of *Campylobacter* spp. was found to be arduous and time consuming. *Campylobacter* characteristic colonies were not always easy to identify and the contaminant microbiota was also frequently observed due to the diversity of colonies present in the plates. In both techniques, chicken samples were proven to be often and widely contaminated, as it was possible to see different types of colonies on both the selective media chosen for the isolation steps (mCCD agar and CFA). Additionally, during re-isolation, *Campylobacter* colonies were very difficult to separate from the background microbiota. By optical microscopy, it was possible to see two types of cells, ones with corkscrew motility and others without characteristic *Campylobacter* morphology. Contaminant microorganisms were generally present after several subsequent re-isolations of a *Campylobacter* characteristic colony on Columbia agar medium or even on selective medium, requiring more purification steps to obtain a pure culture.

Several studies have reported problems in the isolation of *Campylobacter* spp. through ISO's detection and enumeration techniques with the same media chosen for this study (Habib et al., 2008; Seliwiorstow et al., 2016). The increased antimicrobial resistance among *Enterobacteriaceae* during the recent years is indicated to be one of the obstacles. The use of cefoperazone in Bolton broth and mCCD agar as a selective agent to inhibit the growth of contaminant organisms in poultry samples seems to no longer be effective (Belmar Campos et al., 2014; Dierikx et al., 2013; Machado et al., 2008). Jasson et al. (2009) report the ability of extended-spectrum-beta-lactamase (ESBL) producing *E. coli*, which is highly prevalent in broiler meat, to overgrow *Campylobacter* when both organisms are present in the same sample. Baylis et al. (2000) found other competitor organism in poultry meat that is regularly found in enrichment broths of raw meat samples using Bolton broth: *Pseudomonas* spp.. Ghafir et al. (2008) also reports high prevalence of *E. coli* and *Pseudomonas* in Belgium broiler meat. This background microbiota may result in a significant underestimation of the occurrence of *Campylobacter* in the tested matrix (Baylis et al., 2000; Oyarzabal et al., 2013; Rodgers et al., 2010). Thus, the selection of up to five typical colonies as described in the ISO methodology may not be sufficient when other microorganisms are also present (Pinto et al.,

2001). Finally, the methodology and/or culture media for the isolation of *Campylobacter* from food samples needs improvement, bearing in mind the difficulties encountered in the enrichment step and the isolation and purification of the grown bacteria on plates (Jasson et al., 2009).

4.2 Occurrence of *Campylobacter* in kitchen environmental samples

All samples collected before the food preparation proved to be negative for the presence of *Campylobacter* spp.. After food-preparation, six positive samples for *Campylobacter* were obtained, namely: two cutting boards, two sinks and one kitchen cloth, as shown in Table 4. Detailed information on sampling sites performed for each household kitchen is given as supplemental material (Supplemental Tables S1 and S2 – Appendix).

Table 4 – Results for *Campylobacter* spp. detection in cloths, hand towels, sponges and surface samples from 18 Portuguese households.

	Pilot A *	Pilot B	Pilot C *	P01 *	P02 *	P03 *	P04 *	P05	P06	P07 *	P08 *	P09 *	P10 *	P11	P12 *	P13 *	P14 *	P15 *
Chicken rinsing	Yes	Yes	No	Yes	No	Yes	Yes	No	Yes	Yes	No	No	Yes	Yes	Yes	No	Yes	Yes
Cutting board after use	+	-	+	ND	ND	ND	-	-	-	-	-	ND	NA	ND	NA	ND	NA	ND
Cutting board after hand-washing	NA	NA	NA	NA	NA	NA	-	NA	NA	-	NA	NA	NA	NA	NA	NA	NA	NA
Tap handle	-	-	-	-	NA	NA	-	-	-	-	-	-	-	-	-	-	-	-
Handles	-	-	-	-	NA	-	-	-	-	-	-	-	-	-	-	-	-	-
Counter top	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sink	+	-	NA	-	NA	-	-	NA	+	-	NA	NA	-	-	-	NA	-	-
Kitchen Cloth	NA	NA	-	-	-	-	-	-	-	+	-	NA	-	NA	-	-	-	-
Sponge	-	NA	-	-	-	-	-	-	NA	-	-	-	NA	-	-	NA	NA	-
Hand towel	NA	NA	NA	-	NA	NA	NA	-	NA	-	NA	NA	-	NA	NA	NA	NA	NA

NA – Not Applicable; ND – Not determined as the cutting board was only used for vegetables slicing; (+) – positive; (-) – negative; * – chicken meat positive for the presence of *Campylobacter* spp. in at least one of the performed techniques.

Twelve consumers (66.6%) washed the chicken meat under running tap water and eight (44.4%) used cutting boards to prepare the chicken. *Campylobacter* was isolated from the cutting boards of pilot houses A and C, after being used to cut raw chicken; interestingly, *C. jejuni* and *C. coli* strains were isolated from both cutting boards of these houses. The poultry samples collected in these households presented different levels of contamination (i.e. 2.2 x 10^3 CFU/g and present but < 4.0 x 10^1 CFU/g), indicating that even low levels of contamination may contribute to cross-contamination of surfaces during food preparation. In

pilot A, *Campylobacter* was also isolated from the sink. This can be linked to the practice of washing raw poultry before cooking, which was observed during the food preparation session in this household. The sink sample from P06 kitchen was also positive for the presence of this pathogen and, in this case, the consumer also washed the raw poultry. However, *Campylobacter* was undetected in the chicken sample analysed, therefore a direct route of contamination could not be confirmed in this household. One kitchen cloth collected at P07 was also contaminated, and associated to unsafe handling practices confirmed by observational data, namely the direct contact of the cloth with raw poultry.

Overall, two out of the three pilot households presented environmental samples that tested positive for the presence of *Campylobacter*, while only two out of 15 experimental households exhibited positive environmental samples. This can be explained by differences in the sampling methodology applied. In pilot houses an independent swab was used to sample each site of the kitchen for each microbiological parameter included in the study (total viable counts, *Campylobacter* spp., *Salmonella* spp. and/or *L. monocytogenes* enumeration), while in the experimental houses a single sampling cloth was used to sample each site of the kitchen for all microbiological parameters. This resulted in higher detection limits in experimental households' methodology than those observed for pilot houses' microbiological examination (Tables S1 and S2), and thus compromising the detection *Campylobacter* when present in low levels.

From the positive environmental samples, a total of 82 isolates were collected and identified as presumptive *Campylobacter* spp. by phenotypic tests, from which 12 isolates were confirmed to belong to the genus *Campylobacter* (by Multiplex-PCR assay), nine were identified as *C. jejuni* and three as *C. coli*.

4.3. Antimicrobial susceptibility: frequency and patterns

In total, 72 *Campylobacter* isolates (31 *C. jejuni* and 41 *C. coli*) collected from poultry and kitchen's surface and utensils samples were further characterized by phenotypic and genotypic methods. The antimicrobial susceptibility was tested for five classes of antibiotics: β-lactam (ampicillin and ertapenem) and β-lactam conjugated with β-lactamase inhibitor (amoxicillin-clavulanic acid), tetracyclines (tetracycline), macrolides lincosamides and streptogramins (erythromycin), quinolones (ciprofloxacin) and aminoglycosides (gentamicin). Susceptibility was read using zone edges as the point showing no growth viewed from the front of the plate, with reflected light and the lid removed (CASFM & EUCAST, 2017).

Overall, antimicrobial susceptibility testing showed that ciprofloxacin and tetracycline were the antibiotics for which the isolates revealed the highest levels of resistance, with 100% and 94.4% respectively while for amoxicillin-clavulanic acid and gentamicin, the isolates revealed the lowest levels of resistance, 1.4%. Additionally, all *C. jejuni* isolates were susceptible to these two antibiotics. It is important to emphasize that more than 60% of the isolates were resistant to ampicillin.

Campylobacter coli isolates showed higher resistance to all antimicrobial agents than *C. jejuni*. It was further verified that resistance to erythromycin was considerably higher in *C. coli* (65.9%) compared to *C. jejuni* (6.5%) (Table 5).

Table 5 - Antimicrobial drug resistance of *C. jejuni* and *C. coli* isolates from poultry meat and kitchen surfaces and utensils samples

Antimicrobial drug	No. of resistant *C. jejuni* isolates (%)	No. of resistant *C. coli* isolates (%)	Total
Amoxicillin-Clavulanic acid - AMC (20/10µg)	0 (0.0%)	1 (2.4%)	1 (1.4%)
Ertapenem - ETP (10 µg)	1 (3.2%)	5 (12.2%)	6 (8.3%)
Gentamicin - GN (10µg)	0 (0.0%)	1 (2.4%)	1 (1.4%)
Erythromycin - E (15µg)	2 (6.5%)	27 (65.9%)	29 (40.3%)
Tetracycline - TE (30µg)	27 (87.1%)	41 (100%)	68 (94.4%)
Ciprofloxacin - CIP (5µg)	31 (100%)	41 (100%)	72 (100%)
Ampicillin - AMP (10µg)	18 (58.1%)	26 (63.4%)	44 (61.1%)

Combined resistance to antimicrobial agents in these 72 *Campylobacter* spp. isolated from chicken samples and cross-contamination events are presented in Table 6. As mentioned by Duarte et al. (2013) and Magiorakos et al. (2012), a multidrug resistant (MDR) strain is defined to be resistant to at least 3 structurally unrelated antibiotics. Five different antibiotic classes were used in this study and multidrug resistance profiles happened with combined resistance to 3, 4 or 5 classes of antibiotics. Generally, the results show that the majority of the isolates were MDR (63.9%). On the other hand, splitting the results by species, resistance to 2 antibiotics was more common in *C. jejuni* (51.6%) while resistance to 4 antibiotics was more frequent in *C. coli* isolates (53.7%). Thus, 48.4% of the *C. jejuni* isolates can be classified as MDR, while for *C. coli* isolates MDR phenotype was verified in 75.6% of the isolates.

Table 6 - Multidrug resistance (MDR) *C. jejuni* and *C. coli* isolates from poultry meat and kitchen surfaces and utensils samples.

	No. of *C. jejuni* isolates (%)	No. of *C. coli* isolates (%)	Total
Resistant to 2 classes of antibiotics	16 (51.6%)	10 (24.4%)	26 (36.1%)
Resistant to 3 classes of antibiotics	13 (41.9%)	8 (19.5%)	21 (29.2%)
Resistant to 4 classes of antibiotics	2 (6.5%)	22 (53.7%)	24 (33.3%)
Resistant to 5 classes of antibiotics	0 (0%)	1 (2.4%)	1 (1.4%)
Isolates classified as MDR	15 (48.4%)	31 (75.6%)	46 (63.9%)

In EFSA & ECDC (2018a) report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food from 2016, several countries disclosed *Campylobacter* clinical isolates from humans with increasing trends in ciprofloxacin (CIP) and tetracycline (TE) resistance. Overall, *C. jejuni* resistance to erythromycin (E) in EU was low (2.1%), but in *C. coli* strains it was extensively higher with some countries reporting resistance rates ranging from 22.8 to 63.2%. In addition, this report discloses high frequency of combined resistance to CIP, E and TE (Portugal – 47.1%) (EFSA & ECDC, 2018a).

Resistance profiles in isolates from 67 chickens in Portugal showed that all isolates were sensitive to GN (0.6% in human isolates), the occurrence of resistance to CIP and E was higher than in human isolates (95.5% and 10.4% compared to 94% and 6.6% in humans, respectively) and TE resistance was the same as for clinical isolates (82.1% in chickens; 82.0% in humans). In broiler production, tetracyclines were widely used antibiotics because of their low cost, but TE antimicrobial spectrum declined due to resistance mechanisms (Mehdi et al., 2018; Roberts, 1997; Speer et al., 1992). These can be conferred by *tetO* gene, which is commonly plasmid mediated, whose protein confers ribosomal protection due to modification of the TE target (Avrain et al., 2004; Iovine, 2013), or *tetO* gene in synergism with an efflux pump (CmeABC) (Gibreel et al., 2007; Pumbwe & Piddock, 2002).

The results gathered in the present study corroborate the data presented in EFSA & ECDC (2018a) report, except for the resistance to E that was higher than expected. Additionally, and similarly to other studies (Lehtopolku et al., 2010), macrolide-resistant isolates were uniformly multidrug resistant. According to Gibreel & Taylor (2006) and Kim et al. (2006), macrolide resistance is frequent among *Campylobacter* species. This class of antibiotics inhibits protein synthesis in bacteria by binding to the 50S ribosomal subunit, specifically in two domains of the 23S rRNA. Erythromycin resistance in this genus has been associated with a mutation in the 23S rRNA gene (Harrow et al., 2004; Vacher et al., 2003), synergism of this mutation with efflux through CmeABC (Cagliero et al., 2006) and altered

membrane permeability mediated by expression of the major outer membrane porin (MOMP), chromosomally encoded by *porA* (Pumbwe et al., 2004; Wu et al., 2016).

In 2016, nine out of 19 EU member states reported levels of CIP resistance in *C. coli* clinical strains ranging from 80 to 100%. In some countries, this antimicrobial agent can no longer be considered fitting for treatment of campylobacteriosis in humans due to the high level of acquired resistance. The mechanisms that underlie resistance to fluoroquinolones also work synergistically: the modification of CIP target – DNA gyrase and topoisomerase IV – and an efflux pump (CmeABC) (Ge et al., 2005; Luo et al., 2003). In Portugal, the prevalence of CIP resistance in *C. coli* strains is 100%, as observed in this study. Other antimicrobial resistance rates were higher in the present study than the reported number in 2016 (TE - 100% vs. 91.2%; E – 65.9% vs. 50%; GN – 2.4% vs. 0%, respectively) (EFSA & ECDC, 2018a). In addition, and as described by other authors (D'lima et al., 2007; Kim et al., 2006), *C. coli* seems more likely to harbour multiple resistance to antibiotics than *C. jejuni*.

In 1990 in the USA, all *C. jejuni* clinical isolates from humans were susceptible to CIP. However, in 1997–1999 resistance rate rose to 17% and in 2012–2014 reached 25%. This limited the use of fluoroquinolones in the treatment of *Campylobacter* infections, and macrolides were then the drug of choice. On the other hand, resistance to E is increasing among *C. jejuni* isolates, although it has remained low, and it is higher among *C. coli* isolates (Centers for Disease Control and Prevention, 2017).

Bacteria acquire antibiotic resistance by mutation, very common in this genus due to its genome plasticity, and horizontal gene transfer (Jeon et al., 2008). Although *Campylobacter* presents a good restriction modification system, able to decrease the uptake of foreign genetic material, several studies report the acquisition of resistance genes from other microorganisms (Iovine, 2013; Jeon et al., 2008; Nirdnoy et al., 2005; Velázquez et al., 1995). In this study, only one isolate was resistant to GN and few isolates showed resistance to AMC and ETP. β-lactam resistance was more commonly observed as AMP resistance. Possibly, this can be explained by the presence of different types of β-lactamases in synergism with other resistance mechanisms (efflux pumps and decreased membrane permeability due to MOMP) (Alfredson & Korolik, 2005; Iovine, 2013; Lin et al., 2002; Page et al., 1989) but further investigation is needed in this field.

These data reinforce the importance of epidemiological surveillance of this microorganism in Portugal and in the world, since the rising occurrence of MDR strains suggests an increase in their zoonotic potential (Duarte et al., 2013).

4.4. *flaA*-SVR sequencing and PFGE typing of *Campylobacter* spp. and analysis of cross-contamination events

The 72 *Campylobacter* isolates were characterized by performing PFGE and *flaA*-SVR sequencing. Analysis of the DNA sequence variability of the Short Variable Region (SVR) of *flaA* flagellin gene is a useful tool to complement and/or replace other serotyping methods in epidemiologic investigations, at reasonable cost. It is a variation of the *fla* typing method of the entire sequence with a desired twofold redundancy over the entire region, using a pair of forward and reverse primers that bind to conserved flanking sequences (Meinersmann et al., 1997). The *Campylobacter flaA* gene was selected because it is known to be highly variable and a higher discriminant power was observed in the *flaA*-SVR sequencing approach than Restriction Fragment Lenght Polymorphism (RFLP) (Zhang et al., 2018). However, this technique alone is unsuitable as a marker for the molecular epidemiology of *C. coli* and *C. jejuni* (Dingle et al., 2005).

The *flaA* gene amplification, sequencing and analysis were successfully performed for the majority of the isolates tested (n=69). One *C. coli* and two *C. jejuni* isolates' contig analysis were only able to provide a result for the peptide type, but the allele number not identified. This can be due to the selected primers, originally proposed by Meinersmann et al. (1997), that have been previously reported to frequently result in sequences with ambiguous bases (Mellmann et al., 2004; Wassenaar & Newell, 2000), probably because of flagellin gene paralogs (Parkhill et al., 2000), or recombination and intra species transfer. Flagellin A alleles are not very stable and they are not species-specific (Dingle et al., 2005). Figures 3 and 4 show the observed distribution of the isolates' *flaA* allele and peptide numbers. Meinersmann et al. (1997) report as much as a 30% difference in the gene from one isolate to another and this technique identified great diversity of *Campylobacter* spp., which was expected according to the difference in the place of origin. Sixteen different *flaA* types were identified, being the most prevalent allele number 16, followed by number 66. As for *flaA* peptide, lower diversity was observed because of genetic code's redundancy. Eight peptide types were identified, being the most prevalent ones peptide ID 1 and 12.

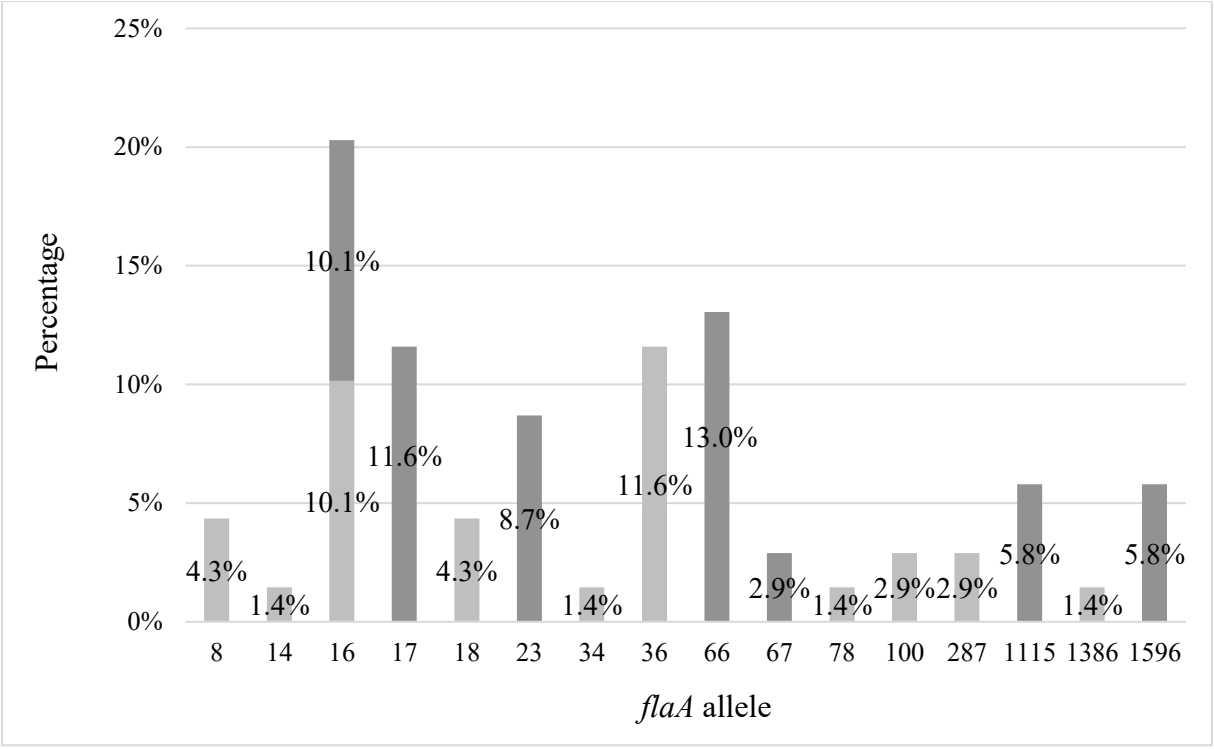


Figure 3 - Occurrence of *flaA* allele identification number in *C. jejuni* (▒) and *C. coli* (▒) isolates from poultry meat and kitchen surfaces and utensils (n=69).

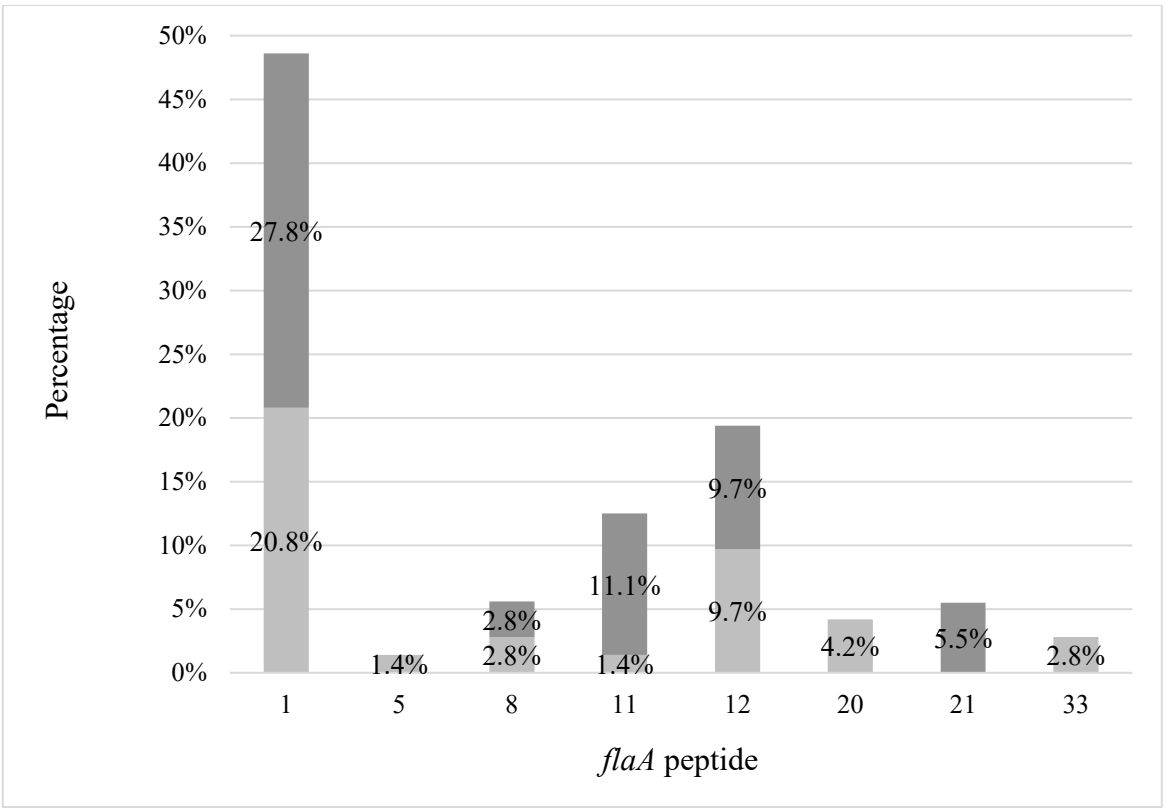


Figure 4 - Occurrence of *flaA* peptide identification number in *C. jejuni* (▒) and *C. coli* (▒) isolates from poultry meat and kitchen surfaces and utensils (n=72).

Pulse-Field Gel Electrophoresis (PFGE) is different from conventional DNA electrophoresis since it can separate large fragments to generate a fingerprint with a specific pattern, by constantly changing the direction of the electric field (https://www.cdc.gov/pulsenet/pathogens/pfge.html). This technique was chosen because it is one of the most robust and suitable typing method for *Campylobacter* spp. (Behringer et al., 2011). PFGE and *flaA*-SVR were combined in order to increase the level of discrimination, since bacterial populations tend to naturally transform along the food chain due to different selection pressures (Elvers et al., 2008; Hyytia-Trees et al., 2007; Newell et al., 2001). A summary of the results obtained in this study is presented in Figure 5.

While Multilocus Sequence Typing (MLST) is considered the "gold standard" for subtyping the majority of *Campylobacter* species and has been widely used (Behringer et al., 2011; Duarte et al., 2016; Ozawa et al., 2016), some authors report as good or even better discriminatory power to PFGE, since it is useful for definition of clones or lineages within *Campylobacter* populations (Clark et al., 2005; On, 2013; On & Harrington, 2001). In this work, two restriction enzymes were selected, *SmaI* and *KpnI*, as the use of a secondary enzyme is always recommended in order to provide a higher discriminatory power to the method (Michaud et al., 2001). In the group of isolates characterized in this study, restriction using *SmaI* and *KpnI* yielded 24 and 27 different patterns, respectively, and, based on combined analysis of both enzyme's patterns, 29 PFGE types were identified, as presented in Figure 5. However, poor additional differentiation was observed between the 72 isolates with the use of *KpnI* as a secondary enzyme, as reported by other studies (Gruntar et al., 2015; Lindmark et al., 2004). Contrarily to *flaA*-SVR sequencing, analysis of PFGE patterns divided *C. coli* (n= 41) and *C. jejuni* (n= 31) isolates into two major independent clusters, revealing a higher genetic diversity among isolates, e.g. *flaA* type 66 isolates present different PFGE patterns. Seventeen unique clusters corresponded to *C. coli* and 12 to *C. jejuni*. Two *C. jejuni* isolates were untypable by *KpnI*, this phenomena has been previously reported by other authors (Gilpin et al., 2006; Oyarzabal et al., 2008). Five *C. jejuni* isolates collected from samples of three households (Pilot A, P09 and P12) exhibited the same PFGE pattern (i.e. pulsotype 002); in these households the chicken meat was bought at two different supermarket chains (A and B). Other two *C. jejuni* isolates from Pilot A household presented 92% of similarity with this cluster. Additionally, two similar *C. coli* isolates exhibited the same PFGE pattern even though the poultry meat from these houses was bought in different supermarkets (i.e. pulsotype 022; households P10 and P15). The remaining 27 PFGE types were unique among the isolates collected from samples at the same household. Household

P02 isolates showed an overall uniform macro-restriction pattern, except for two isolates (D9 and D10) that were isolated from a different method. However, these presented 98.2% of similarity with the remaining isolates as well as the same *flaA* allele and peptide, so the same PFGE type was attributed to all isolates of P02. According to Tenover et al. (1995) isolates are considered to be closely related if their PFGE pattern differ by two to three band, being consistent with a single genetic event, i.e., a point mutation, an insertion or deletion of DNA.

Interestingly, poultry meat samples collected in seven households (Pilot A, P03, P04, P08, P10, P14 and P15) were colonized with more than one *Campylobacter* genotype. This can be explained by the rapid rate of recombination and genomic rearrangements reported within *Campylobacter* genome that hinders the establishment of a population structure and the study of long-term epidemiology (Sails et al., 2003; Wassenaar et al., 1998). Additionally, poultry meat from households Pilot A, Pilot C, P03, P10, P13, P14 and P15 were colonized with both *C. jejuni* and *C. coli*. These results are in accordance with other studies that report a multiple colonization in several flocks analysed (Bull et al., 2006; Hein et al., 2003; Shreeve et al., 2002).

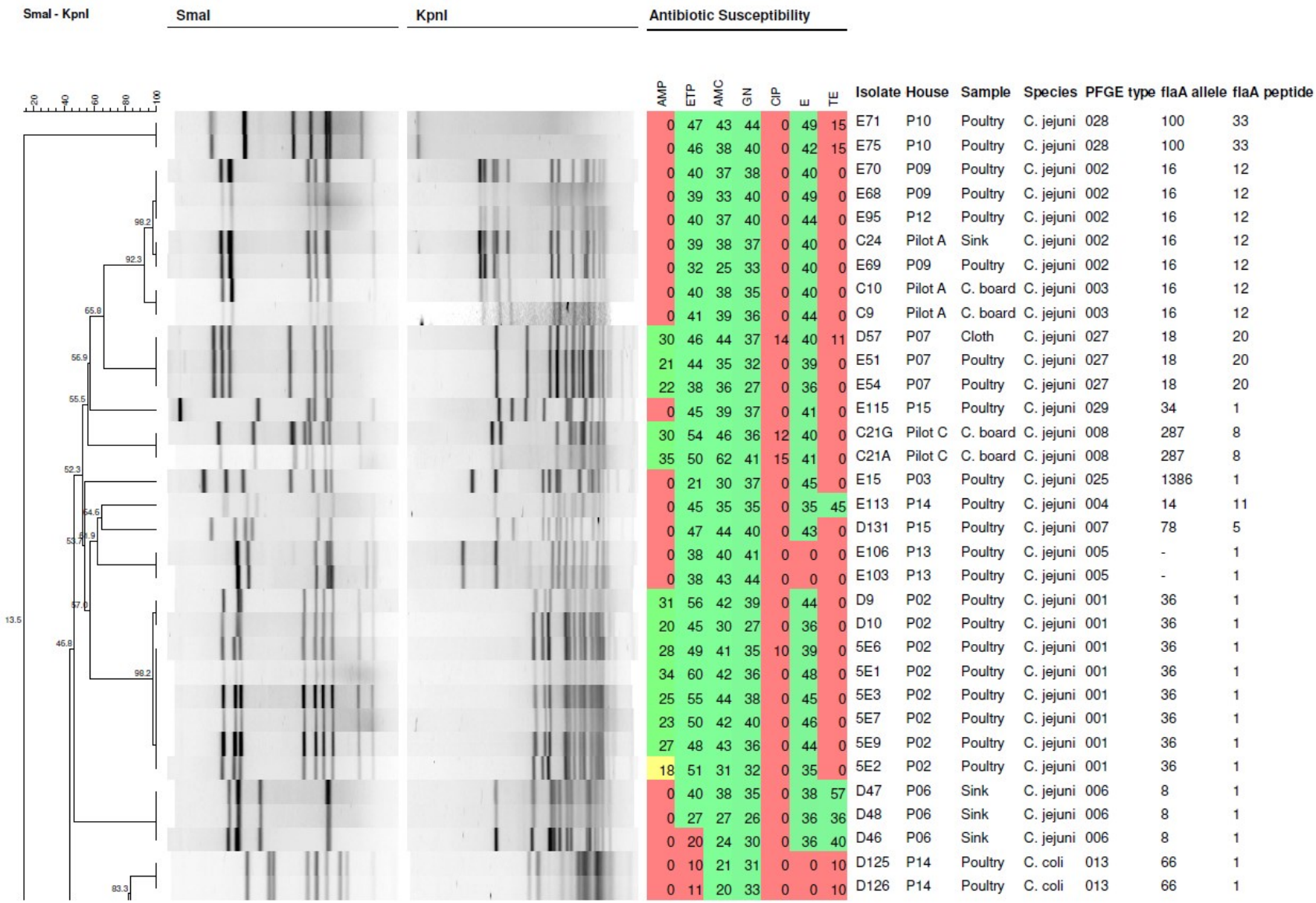
Through the analysis of the typing results obtained combined with observational data collected during food preparation sessions, it is possible to state that cross-contamination events were observed in households Pilot A, Pilot C and P07 via contaminated raw poultry meat to the kitchen environment. Pilot A and Pilot C isolates from the cutting board showed the same PFGE type, *flaA* sequence and peptide number that the one isolated from the poultry meat sample from the respective house. In Pilot A, one *C. jejuni* isolate (C24) was also recovered from the sink sample, and although only *C. coli* isolates were isolated from the raw poultry, this isolate presented the same genotype as *C. jejuni* isolates (C9 and C10) recovered from the cutting board (contaminated both with *C. coli* and *C. jejuni*). Hence it is possible to infer that suspected route of cross-contamination was the raw meat. Similarly, the cutting board of pilot C was contaminated with both *C. jejuni* (C21G and C21A) and *C. coli* (C20), while only *C. coli* isolates were isolated from the raw poultry in this household. In P07, the kitchen cloth was contaminated with the same *C. jejuni* strain found in the raw poultry sample.

In household P06, the sink was found to be the only *Campylobacter* positive site of the kitchen. Three *C. jejuni* isolates were recovered (D47, D48 and D49) and all exhibited the same genotype. As the sample collected before food preparation tested negative for the presence of *Campylobacter* spp. and the raw chicken was washed in the sink during preparation, it is believed that raw chicken was the probable source of contamination.

The pathogen in study was not detected in the meat sample, either because it was not present in the specific chicken part analysed or due to a highly contaminated sample by other species, that render difficult the isolation of characteristics colonies of *Campylobacter*. The hypothesis of the presence of this isolate as a result of a previous contamination episode is very unlikely as the survival and multiplication of this pathogen in the extra-intestinal environment, when exposed to air and light, has been reported to be highly impaired (Cogan et al., 1999; Fernandez et al., 1985). Through comparison of isolation methods, Oyarzabal et al. (2013) concluded that the reference method (ISO 10272) does not capture high variability of strains in a chicken sample, when compared to typing of isolates from other isolation techniques (rinsing of samples in BPW and filtration of the enrichment broth).

Similarly to the present study, other authors reported events of cross-contamination when preparing naturally contaminated chicken meat at home kitchen environment. In a study in Ireland with 12 consumers, cross contamination was reported in 50% of these kitchens, being hands, counter top, oven handle and the draining board positive for the presence of *Campylobacter* (Gorman et al., 2002). In 52 domestic kitchens in the UK, Mattick et al. (2003) reported the survival of this microorganism in 2 out of 52 sponges/dishcloths/scourers and in one out of 32 handtowels during washing-up after preparation of poultry meat with 96% of *Campylobacter* occurrence. Bremer et al. (2005) conducted a survey in private households in Germany inquiring consumers on hygiene in relation to handling various types of raw meat. Respondents reported not cleaning their cutting boards with soap (48.1%) or washing their hands (46.6%) after preparing raw meat. Also in this study, it was noticed that only four of the 15 consumers from the experimental households washed their hands properly, using soap, after handling the raw poultry meat. Luber et al. (2006) quantified the transfer rate of *Campylobacter* spp. during poultry handling. Average cross-contamination rate from hands and kitchen utensils to ready-to-eat food ranged from 2.9% to 27.5%. However, lower percentages were noticed in transfer rates from chicken legs and filets to hands (2.9% and 3.8%), from poultry filets to the cutting board and knife (1.1%) and from chicken legs to the plate (0.3%).

These results highlight the potential for cross contamination and survival of this foodborne pathogen in the kitchen environment and the need to educate the consumer for an appropriate handling of raw chicken meat products.



AMP	ETP	AMC	GN	CIP	E	TE	Isolate	House	Sample	Species	PFGE type	flaA allele	flaA peptide
0	47	43	44	0	49	15	E71	P10	Poultry	C. jejuni	028	100	33
0	46	38	40	0	42	15	E75	P10	Poultry	C. jejuni	028	100	33
0	40	37	38	0	40	0	E70	P09	Poultry	C. jejuni	002	16	12
0	39	33	40	0	49	0	E68	P09	Poultry	C. jejuni	002	16	12
0	40	37	40	0	44	0	E95	P12	Poultry	C. jejuni	002	16	12
0	39	38	37	0	40	0	C24	Pilot A	Sink	C. jejuni	002	16	12
0	32	25	33	0	40	0	E69	P09	Poultry	C. jejuni	002	16	12
0	40	38	35	0	40	0	C10	Pilot A	C. board	C. jejuni	003	16	12
0	41	39	36	0	44	0	C9	Pilot A	C. board	C. jejuni	003	16	12
30	46	44	37	14	40	11	D57	P07	Cloth	C. jejuni	027	18	20
21	44	35	32	0	39	0	E51	P07	Poultry	C. jejuni	027	18	20
22	38	36	27	0	36	0	E54	P07	Poultry	C. jejuni	027	18	20
0	45	39	37	0	41	0	E115	P15	Poultry	C. jejuni	029	34	1
30	54	46	36	12	40	0	C21G	Pilot C	C. board	C. jejuni	008	287	8
35	50	62	41	15	41	0	C21A	Pilot C	C. board	C. jejuni	008	287	8
0	21	30	37	0	45	0	E15	P03	Poultry	C. jejuni	025	1386	1
0	45	35	35	0	35	45	E113	P14	Poultry	C. jejuni	004	14	11
0	47	44	40	0	43	0	D131	P15	Poultry	C. jejuni	007	78	5
0	38	40	41	0	0	0	E106	P13	Poultry	C. jejuni	005	-	1
0	38	43	44	0	0	0	E103	P13	Poultry	C. jejuni	005	-	1
31	56	42	39	0	44	0	D9	P02	Poultry	C. jejuni	001	36	1
20	45	30	27	0	36	0	D10	P02	Poultry	C. jejuni	001	36	1
28	49	41	35	10	39	0	5E6	P02	Poultry	C. jejuni	001	36	1
34	60	42	36	0	48	0	5E1	P02	Poultry	C. jejuni	001	36	1
25	55	44	38	0	45	0	5E3	P02	Poultry	C. jejuni	001	36	1
23	50	42	40	0	46	0	5E7	P02	Poultry	C. jejuni	001	36	1
27	48	43	36	0	44	0	5E9	P02	Poultry	C. jejuni	001	36	1
18	51	31	32	0	35	0	5E2	P02	Poultry	C. jejuni	001	36	1
0	40	38	35	0	38	57	D47	P06	Sink	C. jejuni	006	8	1
0	27	27	26	0	36	36	D48	P06	Sink	C. jejuni	006	8	1
0	20	24	30	0	36	40	D46	P06	Sink	C. jejuni	006	8	1
0	10	21	31	0	0	10	D125	P14	Poultry	C. coli	013	66	1
0	11	20	33	0	0	10	D126	P14	Poultry	C. coli	013	66	1

Figure 5 - Analysis of PFGE using *Sma*I and *Kpn*I restriction enzymes, *flaA*-SVR sequencing and antibiotic susceptibility (in millimetres) of *C. jejuni* and *C. coli* isolates characterized.

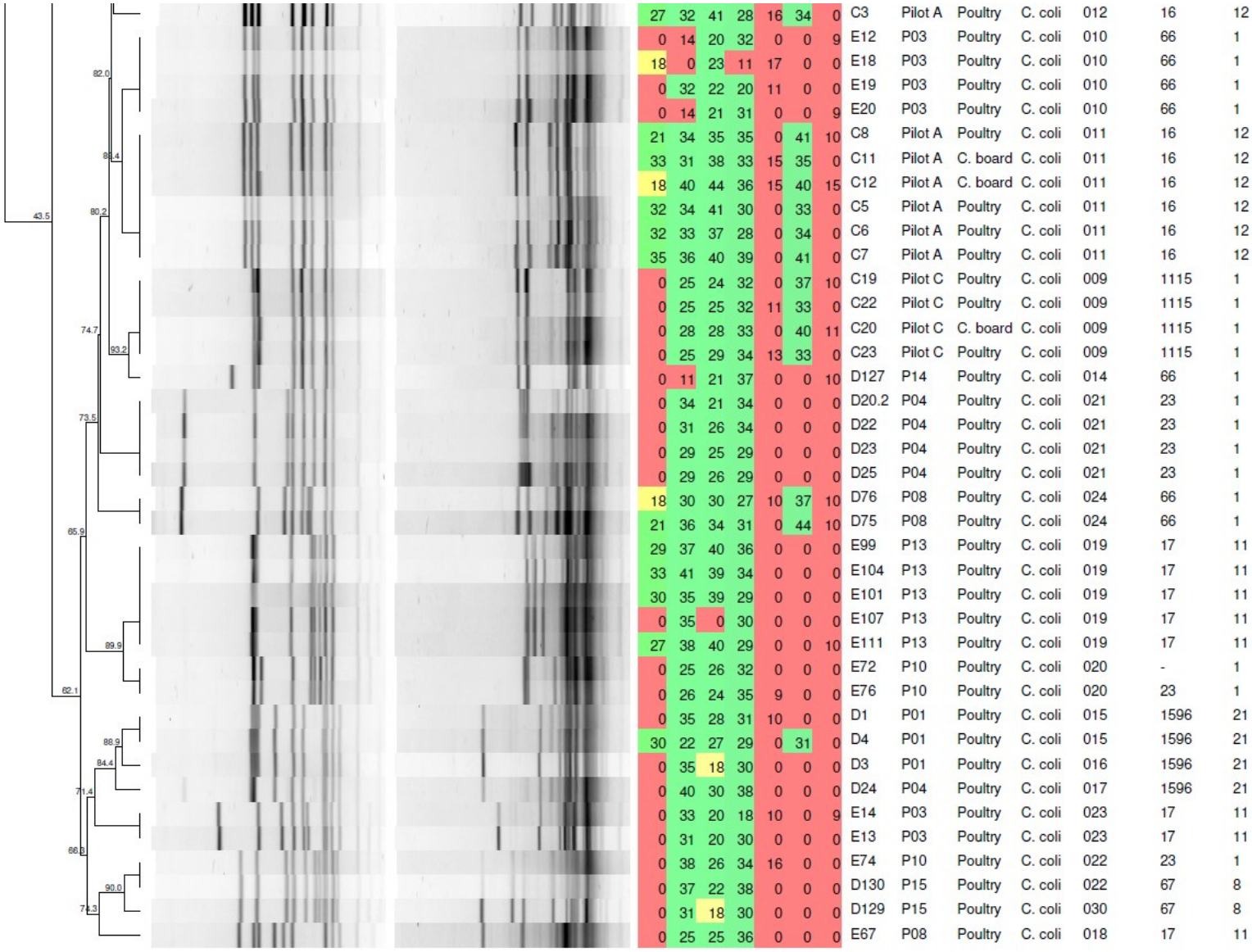


Figure 5 - Analysis of PFGE using *SmaI* and *KpnI* restriction enzymes, f*laA*-SVR sequencing and antibiotic susceptibility (in millimetres) of *C. jejuni* and *C. coli* isolates characterized *(*continuation*)*.

4.5. Genetic diversity of isolates

From the previously mentioned 29 pulsotypes identified in this study, 12 referred to *C. jejuni* isolates and 17 for *C. coli* isolates. The Simpson's index of diversity was calculated for *flaA*-SVR sequencing, restriction with *SmaI*, with *KpnI*, restriction with both enzyme and the combination of PFGE and *flaA*-SVR sequencing, separately for each species. Table 7 shows that the use of a secondary enzyme improved the discriminatory power of the method for *C. coli* isolates, as expected. However, the same result was not observed for *C. jejuni* isolates that obtained the same level of discrimination for both macro-restriction enzymes as well as for PFGE type and the combination of PFGE and *flaA*-SVR results. Higher values of diversity were observed in *C. coli* isolates when PFGE profiles were joint with *flaA* allele's identification. Lower levels of diversity were observed for *flaA*-SVR sequencing alone, for both species.

Table 7 - Simpson's diversity index and confidence intervals (CI) 95% of *C. jejuni* and *C. coli* isolates for each typing method.

	C. jejuni (n= 31)		**C. coli (n=41)**	
	Number of partitions	D (95% CI)	Number of partitions	D (95% CI)
flaA allele	11	0.875 (0.811-0.940)	8	0.862 (0.829-0.896)
SmaI type	12	0.897 (0.837-0.957)	12	0.891 (0.837-0.946)
KpnI type	12	0.897 (0.837-0.957)	15	0.928 (0.901-0.955)
PFGE type (*SmaI* + *KpnI*)	12	0.897 (0.837-0.957)	17	0.940 (0.915-0.966)
PFGE type + *flaA* allele	12	0.897 (0.837-0.957)	19	0.943 (0.916-0.970)

CI – Confidence interval

5. Conclusion

Campylobacter spp. was isolated from 77.8% of the raw poultry meat tested at least by one of the methods applied (enumeration and/or detection). Of the 18 samples analysed, only one exceeded the limit of 1,000 CFU/g determined by Regulation (EC) No 2017/1495. Cross-contamination events between contaminated raw poultry meat and the environment (i.e. cutting board, sink or kitchen cloth) were identified in four kitchens. Both *C. jejuni* and *C. coli* were recovered from poultry and these surfaces/utensils. The difficulties in the isolation of this microorganism through traditional methodologies can imply an even higher incidence of *Campylobacter* spp. in the analysed samples.

Antimicrobial resistance showed rising occurrence of MDR strains, with 63.9% of the isolates being resistant to more than 3 classes of antibiotics. All the isolates recovered from consumers' households were resistant to ciprofloxacin and 94.4% were resistant to tetracycline. Higher resistance to antibiotics was shown by *C. coli* than *C. jejuni* isolates. Typing of *Campylobacter* isolates originated 29 pulsotypes and 16 *flaA* alleles with eight different *flaA* peptide identifications that allowed to confirm raw chicken as potential source of cross-contamination.

These results highlight the potential for cross-contamination and survival of this foodborne pathogen in the kitchen environment and the need to educate the consumer for appropriate handling of raw chicken meat products.

6. Future work

The results showed in this work should be further explored. Thus, some interesting subjects are suggested:

- Expansion of this work to other regions of Portugal, to meet other food handling practices along the country;
- Study of the prevalence of *Campylobacter* spp. in chicken meat at retail level in Portugal;
- Genotypic characterization of *Campylobacter* spp. isolates circulating from farm to fork in Portugal;
- Study of the transfer rate of *Campylobacter* during preparation of naturally contaminated poultry to hands and kitchen environment;
- Study of antimicrobial resistance mechanisms present in these isolates and other isolates from poultry meat sold in other regions of Portugal;
- Genotypic comparison of these food and cross-contamination isolates with clinical isolates from patients with campylobacteriosis;
- Detection of *Campylobacter* spp. in kitchen and hand samples though methods that can detect viable not culturable cells (Real time-PCR with specific *Campylobacter* primers of 16S rRNA and specific most common species genes);
- Study of virulence, adhesion and invasion of cross contamination isolates compared to chicken isolates.

Appendix

Supplemental Table S1. Detection limits (DL) for each sampling site collected at different households.

Household		Sampling Site[a]				
		Cutting board	**Tap handle**	**Counter top**	**Sink**	**Handles**
Pilots A, B and C	Sampling area	Whole area	Whole area	100 cm^2	Whole area	6 handles
	DL	1 cell/cutting board	1 cell/tap handle	1 cell/100 cm^2	1 cell/sink	0.2 cells/handle
P01	Sampling area	726.0 cm^2	100 cm^2	2064 cm^2	12493 cm^2	7 handles
	DL [b]	1 cell/29.0 cm^2	1 cell/4.0 cm^2	1 cell/82.6 cm^2	1 cell/499.7 cm^2	1 cell/0.3 handle
		3.4 cells/100 cm^2	25.0 cells/100 cm^2	1.2 cells/100 cm^2	0.2 cells/100 cm^2	3.6 cells/handle
P02	Sampling area	630.0 cm^2	471.2 cm^2	2820 cm^2	3564 cm^2	6 handles
	DL [b]	1 cell/25.2 cm^2	1 cell/18.8 cm^2	1 cell/112.8 cm^2	1 cell/142.6 cm^2	1 cell/0.2 handle
		4.0 cells/100 cm^2	5.3 cells/100 cm^2	0.9 cells/100 cm^2	0.7 cells/100 cm^2	4.2 cells/handle
P03	Sampling area	459.0 cm^2	362.9 cm^2	2240 cm^2	3440 cm^2	8 handles
	DL [b]	1 cell/18.4 cm^2	1 cell/14.5 cm^2	1 cell/89.6 cm^2	1 cell/137.6 cm^2	1 cell/0.3 handle
		5.4 cells/100 cm^2	6.9 cells/100 cm^2	1.1 cells/100 cm^2	0.7 cells/100 cm^2	3.1 cells/handle
P04	Sampling area	825.0 cm^2	340.9 cm^2	2400 cm^2	2610 cm^2	6 handles
	DL [b]	1 cell/33.0 cm^2	1 cell/13.6 cm^2	1 cell/96.0 cm^2	1 cell/104.4 cm^2	1 cell/0.2 handle
		3.0 cells/100 cm^2	7.3 cells/100 cm^2	1.0 cells/100 cm^2	1.0 cells/100 cm^2	4.2 cells/handle
P05	Sampling area	644.0 cm^2	340.8 cm^2	680 cm^2	3068 cm^2	6 handles
	DL [b]	1 cell/ 25.8 cm^2	1 cell/13.6 cm^2	1 cell/27.2 cm^2	1 cell/122.7 cm^2	1 cell/0.2 handle
		3.9 cells/100 cm^2	7.3 cells/100 cm^2	3.7 cells/100 cm^2	0.8 cells/100 cm^2	4.2 cells/handle
P06	Sampling area	644.0 cm^2	133.5 cm^2	2000 cm^2	4240 cm^2	6 handles
	DL [b]	1 cell/25.8 cm^2	1 cell/5.3 cm^2	1 cell/80.0 cm^2	1 cell/169.6 cm^2	1 cell/0.2 handle
		3.9 cells/100 cm^2	18.7 cells/100 cm^2	1.3 cells/100 cm^2	0.6 cells/100 cm^2	4.2 cells/handle
P07	Sampling area	638.0 cm^2	638.0 cm^2	133.5 cm^2	2000 cm^2	5 handles
	DL [b]	1 cell/25.5 cm^2	1 cell/25.5 cm^2	1 cell/5.3 cm^2	1 cell/80.0 cm^2	1 cell/0.2 handle
		3.9 cells/100 cm^2	3.9 cells/100 cm^2	18.7 cells/100 cm^2	1.3 cells/100 cm^2	5.0 cells/handle
P08	Sampling area	750.0 cm^2	282.7 cm^2	1000 cm^2	2870 cm^2	7 handles
	DL [b]	1 cell/30.0 cm^2	1 cell/11.3 cm^2	1 cell/40.0 cm^2	1 cell/114.8 cm^2	1 cell/0.3 handle
		3.3 cells/100 cm^2	8.8 cells/100 cm^2	2.5 cells/100 cm^2	0.9 cells/100 cm^2	3.6 cells/handle

Supplemental Table S1. (continuation)

Household		Sampling Site[a] Cutting board	Tap handle	Counter top	Sink	Handles
P09	Sampling area	805.0 cm^2	362.9 cm^2	1400 cm^2	3303 cm^2	6 handles
	DL [b]	1 cell/32.2 cm^2	1 cell/14.5 cm^2	1 cell/56.0 cm^2	1 cell/132.1 cm^2	1 cell/0.2 handle
		3.1 cells/100 cm^2	6.9 cells/100 cm^2	1.8 cells/100 cm^2	0.8 cells/100 cm^2	4.2 cells/handle
P10	Sampling area	-	263.9 cm^2	1200 cm^2	2580 cm^2	6 handles
	DL [b]	-	1 cell/10.6	1 cell/48.0	1 cell/103.2	1 cell/0.2 handle
			9.5 cells/100 cm^2	2.1 cells/100 cm^2	1.0 cells/100 cm^2	4.2 cells/handle
P11	Sampling area	660.0 cm^2	164.9 cm^2	1200 cm^2	2396 cm^2	7 handles
	DL [b]	1 cell/26.4 cm^2	1 cell/6.6 cm^2	1 cell/48.0 cm^2	1 cell/95.8 cm^2	1 cell/0.3 handle
		3.8 cells/100 cm^2	15.2 cells/100 cm^2	2.1 cells/100 cm^2	1.0 cells/100 cm^2	3.6 cells/handle
P12	Sampling area	-	196.4 cm^2	1200 cm^2	3308 cm^2	7 handles
	DL [b]	-	7.9	48.0	132.3	1 cell/0.3 handle
			12.7	2.1	0.8	3.6 cells/handle
P13	Sampling area	750.0 cm^2	87.9 cm^2	900.0 cm^2	3325 cm^2	5 handles
	DL [b]	1 cell/30.0 cm^2	1 cell/3.5 cm^2	1 cell/36.0 cm^2	1 cell/133.0 cm^2	1 cell/0.2 handle
		3.3 cells/100 cm^2	28.4 cells/100 cm^2	2.8 cells/100 cm^2	0.8 cells/100 cm^2	5.0 cells/handle
P14	Sampling area	-	263.9 cm^2	1200 cm^2	2760 cm^2	9 handles
	DL [b]	-	1 cell/10.6 cm^2	1 cell/48.0 cm^2	1 cell/110.4 cm^2	1 cell/0.4 handle
			9.5 cells/100 cm^2	2.1 cells/100 cm^2	0.9 cells/100 cm^2	2.8 cells/handle
P15	Sampling area	340.0 cm^2	243.5 cm^2	1800 cm^2	3810 cm^2	5 handles
	DL [b]	1 cell/13.6 cm^2	1 cell/9.7 cm^2	1 cell/72.0 cm^2	1 cell/152.4 cm^2	1 cell/0.2 handle
		7.4 cells/100 cm^2	10.3 cells/100 cm^2	1.4 cells/100 cm^2	0.7 cells/100 cm^2	5.0 cells/handle

[a] Sampling site swabs from the pilot households were homogenised with 10 ml of sterile Bolton broth with 5% defibrinated horse blood in the stomacher for 1 minute and incubated at 41.5 ºC under microaerophilic conditions for 48h; whereas swabbing cloths from the experimental households were homogenised with 25 ml of sterile buffered peptone water (BPW) in a stomacher for 1 minute, and subsequently a 1 ml aliquot of the homogenate was inoculated into 9 ml Bolton broth tube with 5% defibrinated horse blood, and incubated in the same conditions.

[b] In the experimental households the detection limit (cell/100 cm^2) for each sampling site was calculated using the following equation: DL= 100 / [1/25 * A], where A=sampling area.

Supplemental Table S2. Detection limits (DL) for the kitchen utensils site collected at the pilots and experimental households.

Sampling item	**Dilution Volume (ml)**	**Detection Limit (1 cell/ *x* item)**	**Tap handle cells/item**
Kitchen sponge	50	0.02	50.0 cells/sponge
Kitchen cloth	25	0.004	225.0 cells/cloth
Hand Towel	225	0.04	25.0 cells/hand towel

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