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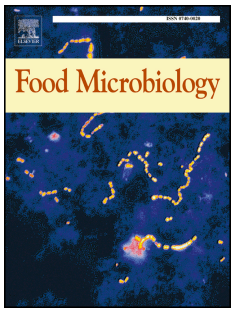

Characterization of *Campylobacter* species in Spanish retail from different fresh chicken products and their antimicrobial resistance

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Title: Characterization of *Campylobacter* species in Spanish retail from different fresh chicken products and their antimicrobial resistance.

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Abstract

Contaminated chicken products have been recognized as the primary vehicles of *Campylobacter* transmission to human. Pulsed-field gel electrophoresis (PFGE) and antimicrobial resistance of *Campylobacter* isolates from fresh chicken products at retail were studied. A total of 512 samples including: thigh, breast, marinated and minced chicken were purchased from different retail stores. Half of the samples were packed and the other half were unpacked. The 39.4 % of the samples were *Campylobacter* positive; being unpacked chicken products (45.3 %) more contaminated than packed chicken (33.6 %). PFGE typing showed a high diversity among isolates; clustering 204 isolates into 76 PFGE types: 55 clusters of *C. jejuni*, 19 of *C. coli* and 2 of *C. lari. C. coli* genotypes showed higher resistance than other *Campylobacter* species. Although modified atmosphere packaging can reduce the prevalence of *Campylobacter* spp., it does not avoid their presence in at least 33.6 % of packed chicken products analyzed. Some pulsotypes might persist in the processing plant or butcher shops environment for longer than previously thought. More stringent control measures are needed in previous steps of the chicken food chain, in order to avoid the presence of *Campylobacter* spp. strains at retail that can compromise consumer´s safety.

Keywords: *Campylobacter* spp., PFGE, antibiotic resistance, persistence.

1. Introduction

Campylobacteriosis has been reported as the most common zoonosis, with an increase in confirmed human cases in the European Union since 2008 (EFSA, 2017). Large outbreaks are uncommon and the vast majority of human campylobacteriosis cases are sporadic (Pires et al., 2010). Most *Campylobacter* infections are self-limiting and do not require treatment with antimicrobials. However, severe and prolonged cases of campylobacteriosis and infections in immune-compromised, vulnerable populations and children may require antimicrobial therapy. In these cases, fluoroquinolones and macrolides such as erythromycin are the drugs of choice (Narváez-Bravo et al., 2017).

Several foodstuffs have been involved in the transmission of the pathogen to humans; such as, untreated water (Nilsson et al., 2017; Revez et al., 2014), milk and cheese (EFSA, 2017), salad (Calciati et al., 2012), spinach, lettuce, radish, green onion, potatoes, parsley (Park and Sanders, 1992) and fenugreek (Kumar et al., 2001). However, poultry has been identified as the natural host for *Campylobacter* species, and broilers are often colonized, especially with *C. jejuni* (Sasaki et al., 2011; Torralbo et al., 2014). Contamination of broiler flocks at farm level, often lead to transmission of *Campylobacter* along the poultry production chain and contamination of poultry meat at retail (Melero et al., 2012; Skarp et al., 2016). The role of poultry as a reservoir for this transmission has been recognized with 20-30 % of the human infections linked to handling, preparation, and consumption of broiler meat (EFSA, 2010).

C. jejuni has traditionally been categorized as a fastidious microorganism by its metabolic features, it is an asaccharolytic microorganism (it has limitations in the utilization of hexose sugars) and is considered to be microaerophilic and capnophilic, because requires both O_2 and CO_2 for optimal growth, preferably at 5–10 % and 1–10 %, respectively (Bolton and Coates, 1983; Oh et al., 2017). However, it could survive in harsh environmental conditions, such as surfaces and equipment in broiler's slaughterhouse (García-Sánchez et al., 2017), poultry products in aerobic conditions (Di Giannatale et al., 2014) and modified atmosphere packaging (Melero et al., 2013; Meredith et al., 2014). *Campylobacter* could be recovered along all steps in the transmission through the food chain (Melero et al., 2012). Oxidative stress is one of the first obstacles that *Campylobacte*r has to face in the extra-intestinal environment (Atack and Kelly, 2009). Therefore, reduced sensitivity of some strains to oxygen would confer superior environmental resistance, increasing the likelihood of transmission between potential hosts (O´Kane et al., 2017).

Recent studies have shown a high percentage of *Campylobacter* in broiler fresh meat (36.7 %) in comparison with other poultry fresh products in the EU Member States, corresponding the highest

country-specific notification rates in 2016 to Czech Republic, Slovakia, Sweden and the United Kingdom (EFSA, 2017). Additionally, studies done by the Food Standards Agency corroborated that more than half of fresh chicken products, bought in retail shops and produced in UK, tested positive for *Campylobacter* in the period between 2016 and 2017 (Whitworth, 2017). In that sense, evaluation of poultry meats at retail is critical, as they really enter the consumers' kitchens (Cook et al., 2012). Cross-contamination occurs between fresh chicken meat and consumer's kitchen utensils and hands and ready-to-eat products at home (Luber et al., 2006).

The aim of this study was (i) to investigate the prevalence and genotypic profile of *Campylobacter* species in different chicken products in Spanish markets and (ii) to determine the antimicrobial Minimum Inhibitory Concentration (MIC) in different *Campylobacter* spp. populations found in retail shops.

2. Material and methods

2.1. Study area and sampling procedure

The study area was located in the city of Burgos in the North of Spain (107 km²) which has a total of 177,100 inhabitants. The study was carried out from 23 th February to 18 th June in 2015. During this period, a total of 512 chicken samples were purchased from 18 retail shops: 12 butcher shops and 6 markets. Thigh (n=128) breast (n=128), minced (n=128) and marinated (n=128) fresh chicken products were analyzed. Marinated products contained several spices such as paprika, garlic, oregano and, in some cases, wine. Half of the samples (n=256) were purchased in markets and correspond to products packed with modified atmosphere (MAP), whereas the other half (n=256) were purchased in butcher shops in bulk without packing (aerobic or ambient atmosphere).

During this period, 13 different suppliers (A-M) of fresh chicken packed products were sampled. These were classified according to their geographical origin in four regions: East (E), West (W), North (N), and Centre (C) (Figure 1). However, unpacked products were purchased in local butcher shops (a-l) located around the city. From these, at least four of them (a, b, c, d) received meat from the same slaughterhouse.

2.2. Gas and pH analyses

In MAP fresh chicken products, gas analysis was carried out with a digital O_2/CO_2 analyzer (OXYBABY, WITT-Gasetechnik GmbH & Co KG, Witten, Germany). Ten milliliters gas samples were drawn from the pack headspace by the needle of the analyzer through a septum glued onto the surface of the pack.

Once the pack was opened, pH was measured in the product. A pH meter (micropH2001, CRISON, Barcelona, Spain) was used by inserting the pin electrode directly into the sample.

Gas and pH measures were carried out in each product in triplicate.

2.3. Isolation and identification of *Campylobacter* spp.

From each sample, 10 g were aseptically taken and placed in sterile stomacher bags for homogenization with 90 ml of sterile Preston broth made with Nutrient Broth Nº 2 (Oxoid, Basingstoke, England) supplemented with Preston Campylobacter Selective Supplement (Oxoid) and Campylobacter Growth Supplement Liquid (Oxoid). Each sample was individually homogenized with Preston broth for 120 s and incubated microaerobically using a commercial gas-generating system CampyGen (Oxoid) and sealed jar at 41.5 ºC for 48 h. After enrichment, a loop-full from each sample was streaked on a plate of modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) prepared with Campylobacter blood-free selective agar base (Oxoid) supplemented with CCDA selective supplement (SR0155E, Oxoid). Plates were incubated as described above for enrichment broths. From each plate, two typical isolated *Campylobacter* spp. colonies were randomly selected for further analysis.

Isolated colonies from the mCCDA agar were grown on 5 mL of Brain Heart Infusion broth (Oxoid) overnight. DNA was extracted according to Yamada et al. (2015). Briefly, strains were suspended in 100 µL of Tris-EDTA buffer (pH 8.0) and incubated at 95 ºC for 10 min, and centrifuged at 16,000×*g* for 1 min. The supernatants were subsequently used as templates for PCR. All isolates were analysed using multiplex PCR to identify *C. jejuni, C. coli, C. lari, C. upsaliensis* and *C. fetus sub. fetus* as described by Wang et al. (2002). Gels were stained with ethidium bromide solution and photographed with Gel Doc XR System (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

2.4. Pulsed field gel electrophoresis (PFGE)

Campylobacter spp. isolates were cultured on Columbia agar (Oxoid) supplemented with 5 % defribrinated sheep blood (Oxoid) under microaerobic conditions (24 h at 41.5 ºC) for the purpose of typing. PFGE analyses were performed following the protocol according to PulseNet (www.cdc.gov/pulsenet/pathogens/pfge.html) and applying the restriction enzymes *Sma*I and *Kpn*I. *Kpn*I was used to check the diversity of all isolates with similar *Sma*I genotype.

Restricted DNA was electrophoresed for 22.5 h on 1 % (w/v) SeaKem gold agarose in 0.5 × TBE at 6 V/cm on a Chef DR III system (Bio-Rad Laboratories). The electrophoresis conditions used consisted of an initial switch time of 5 s and a final switch time of 55 s (gradient of 6 V/cm and an

included angle 120º). Gels were stained with ethidium bromide solution and photographed with Gel Doc XR System (Bio-Rad Laboratories). BioNumerics 6.5 (Applied Maths, Sint-Martens-Latem, Belgium) was used for numerical analysis of *Sma*I and *Kpn*I macrorestriction patterns. Similarity analysis was carried out using the Dice coefficient (position tolerance, 1.0 %). The unweighted pair-group method using arithmetic averages (UPGMA) was used to cluster patterns. Isolates with <85 % similarity according to the dendrogram were clustered as separate pulsotypes (Boer et al., 2000).

2.5. Antimicrobial susceptibility

Campylobacter spp. isolates were sub-cultured on Nutrient Agar supplemented with 5% sheep blood (Oxoid) and incubated at 41.5 ºC for 24 h in microaerophilic conditions. After incubation, bacterial inoculum was introduced into 2 mL of 0.9 % NaCl and the turbidity was adjusted to 0.5 McFarland scale to carry out the inoculation. The inoculated plates were incubated in microaerophilic conditions for 48 h at 41.5 ºC. Six antimicrobials belonging to four different classes were tested in different range concentrations: two fluoro(quinolones): ciprofloxacin (0.03-64 mg/L) and nalidixic acid (4-128 mg/L); two macrolides: erythromycin (0.12-16 mg/L) and azithromycin (0.015-1 mg/L); one aminoglycoside: gentamicin (0.12-8 mg/L) and tetracycline (0.12-128 mg/L). Isolates were considered to be susceptible or resistant based on epidemiological cutoff values according to European Committee on Antimicrobial Susceptibility Testing (www.eucast.org). One isolate was considered multi-drug resistance, when it was resistant to three or more unrelated antimicrobials. Strain *C. jejuni* ATCC 33560™ was used as a control.

3. Results

3.1. Gas and pH analyses results

In packed fresh chicken products, atmosphere composition and pH were measured. As it can be observed in table 1, *Campylobacter* strains were isolated from samples with a pH ranging between 5.9-6.5 and gas composition between 11.4-77.5 % of O_2 and between 1.5 and 53.9 % of CO_2. Only in four out of 15 suppliers *Campylobacter* spp. was absent.

3.2. Prevalence of *Campylobacter* spp. in chicken meat

A total of 202 out of 512 samples were *Campylobacter* spp. positive (39.4%). A higher prevalence (45.3 %) was observed in unpacked products compared to packaged products, which was 33.6 %. The most contaminated products were the unpacked marinated products from butcheries and packed thighs from markets with the same percentage (56.3%), followed by 51.6 % in thigh and breast (unpacked). Minced products in both atmospheres were the least contaminated with a percentage of 21.9 % and 14 % in unpacked and packed products, respectively (Table 2). In general, in packed

157 products most of the strains were isolated from thigh and breast products, whereas in unpacked
158 products isolates came from all different chicken products.

159 *C. jejuni* was the most prevalent species in all products analyzed. *C. jejuni* accounted for 77.7 % of
160 total positive products (157/202), followed by *C. coli* 16.3 % (33/202) and *C. lari* 2.5 % (5/202). A
161 low percentage of positives samples (3.5 %) showed a mix of species in the same product, the
162 majority of them were a mix of *C. jejuni/C. coli* except in one that was *C. jejuni/C. lari*. Among
163 unpacked product, *C. jejuni* represented 87.1 % of positives samples, followed by *C. coli* (11.2 %)
164 and *C. lari* (1.7 %). However, in packed products *C. coli* were present in a higher percentage
165 (23.3%) in several cases, taking part of the mix of species together with *C. jejuni* (Table 2).

166 **3.3. *Campylobacter* spp. genotyping**

167 From the two typical colonies isolated per product, only one in cases where both belonged to the
168 same species was typed by PFGE. On the contrary, in those products in which a mix of two
169 different species was found, both isolates were typed. Therefore, 164 isolates from *C. jejuni*, 39
170 from *C. coli* and 6 from *C. lari* were selected. From these, 5 isolates were lost during the typing
171 process (2 *C. jejuni*, 2 *C. coli* and 1 *C. lari*).

172 Pulse field gel electrophoresis clustered the 204 *Campylobacter* spp. isolates into 76 PFGE profiles.
173 Among them, 55 PFGE types correspond to *C. jejuni* (162 isolates), 19 pulsotypes to *C. coli* (37
174 isolates) and 2 types to *C. lari* (5 isolates) as shown in figures 2 and 3 (see complete dendrogram in
175 supplementary information). The majority of clusters were formed by one or two isolates, showing
176 a wide diversity among chicken products. From the total of 55 *C. jejuni* types, 28 clusters (50.9 %)
177 included one isolate, similar situation occurs in *C. coli* where 52.6 % corresponds to clusters with
178 one isolate only.

179 In general terms, unpacked products have shown less PFGE types although with higher numbers of
180 isolates (9 unpacked products with 2 to 22 isolates forming the different clusters). However, packed
181 products have shown more PFGE types although with less number of isolates (11 products from 2
182 to 7 isolated harbored the cluster). PFGE types sharing isolates from unpacked and packed chicken
183 products were represented by 6 clusters with a range of 5 to11 isolates.

184 Clusters which harbored more than 3 isolates in the case of *C. jejuni* and *C. lari* and more than 2 in
185 *C. coli* are shown in table 3. PFGE j-44 was the most prevalent within *C. jejuni* population (13.5
186 %). Isolates from this pulsotype were present only in unpacked products which were purchased in
187 local butcher shops. The same situation occurred with type j-54 and j-37 with 6.8 % and 5.5 %

respectively. The PFGE type j-44 with 22 isolates together with j-8 and j-54, both with eleven isolates, were recovered during approximately 1 month in different butcher shops. A similar situation happens with cluster j-37 that was isolated during longer time, around two months. In both cases, most of the strains were isolated from samples coming from the local slaughterhouse. Moreover, j-24, j-30, j-31, j-47 and j-49 were recovered from packed and unpacked chicken products along more than 2 and even 3 months (Table 3).

Pulsotype j-10 comprises strains from two different geographical origins from packed chicken products (C-West and I-Center). However, isolates from C-West origin appeared in samples taken with a difference of one month and a half between them. The same situation occurs with cluster c-14 from *C. coli* (c-14) appearing isolates with a difference around two months in packed products from the same supplier E2 (Center). Moreover, in this case, both strains were isolated from the same type of chicken products: thigh. Additionally, *C. lari* (type l-2) was recovered from the same supplier E1 (North) with a difference of 25 days.

3.4. Antibiotics resistance

The phenotypic antimicrobial susceptibility determined for the 76 types corresponding to 55 *C. jejuni* (from j-1 to j-55), 19 *C. coli* (from c-1 to c-19) and 2 *C. lari* (l-1 and l-2) is shown in tables 4 and 5. *C. jejuni* pulsotypes were 100 % resistant to (fluoro)quinolones, 98.2 % to tetracycline and 1.8 % to azithromycin. However, some differences were observed in types j-19 and j-21; being j-19 resistant to azithromycin and j-21 sensitive to tetracycline (Table 4). Similar situation occurred with all *C. lari* pulsotypes (l-1, l-2), being resistant to quinolones and tetracycline (100 %) and l-1 showing as well resistance to gentamycin (Table 5).

Strains of *C. coli* were more resistant (Table 5). All strains were resistant to tetracycline (100%). Regarding to (fluoro)quinolones, all strains showed more resistance to nalidixic acid (100 %) than to ciprofloxacin (78.9 %). Resistance to both macrolides (erythromycin and azithromycin) was 52.6 %. Only pulsotype c-19 showed resistance to gentamycin. According to these results, one pulsotype of *C. jejuni* (j-19), nine of *C. coli* (c-2, c-3, c-4, c-8, c-9, c-10, c-12, c-17 and c-19) and one *C. lari* (l-1) can be considered multidrug-resistant.

4. Discussion

Monitoring *Campylobacter* prevalence and population typing in chicken products at retail level is important to assess a potential human health risk and to explore possible interventions to reduce it. Transmission along the broiler meat supply chain has been established as the main source of *Campylobacter* contamination in humans. For this reason, it is important to collect as much

information as possible, to prevent and design strategies to control the presence of *Campylobacter*. According to our results the 39.4 % of chicken samples analyzed harbored *Campylobacter*. EFSA studies have shown similar percentages (39.99 %) in fresh meat from broiler at retail in European Union, reaching in Spain 50 % (EFSA, 2017).

Among the total analyzed products, packed chicken (MAP) presented a lower prevalence (33.6 %) than unpacked products (45.3 %). These results agree with other studies, where *Campylobacter* was less present in MAP than in other chicken products stored under ambient or vacuum (Luber and Bartelt, 2007). How different gas concentrations used in MAP might affect *Campylobacter* survival is not yet fully understood. Due to the microaerophilic nature of *Campylobacter*, some authors state that high concentrations of more than 70 % O_2 reduce *Campylobacter* spp. counts in more than two logs (Boysen et al., 2007; Rajkovic et al., 2010). Although, it seems that those concentrations favor the growth of meat spoilage bacteria. On the contrary, other authors found some aerotolerant or even do hyper-tolerant *Campylobacter* strains able to grow in high O_2 concentrations on the culture media, suggesting that high CO_2 atmospheres can reduce or inhibit the presence of *Campylobacter* in chicken packed products (Oh et al., 2017). Moreover, Meredith et al. (2014) recommended a gas composition of 40 CO_2/30 O_2/30 N_2 as the most appropriate gaseous mixture for achieving the dual objective of extending shelf-life while inhibiting *Campylobacter* survival. In this study, *Campylobacter* strains have been isolated from packages with a broad range of O_2 (11.4-77.5 %) and CO_2 (1.5-53.9 %) composition. This fact indicates that MAP might have a positive effect against *Campylobacter*, although it is not enough to eliminate the pathogen in these products, suggesting that these strains may have greater resistance to O_2 and CO_2 than previously thought. Therefore, pre-harvest measures as biosecurity and/or post-harvest ones, such as scalding, chilling and removal of fecal residues might be considered before packing poultry products (Osimani et al., 2017).

Unpacked products presented higher *Campylobacter* spp. contamination (45.3 %). All butcher shops were positive to *Campylobacter* in each sampling day, as well as in all the products types analyzed although with some variations. Due to the fastidiousness and oxygen sensitivity, *C. jejuni* is not expected to survive efficiently during foodborne transmission in oxygen-rich, atmospheric conditions. However, our results evidenced that the pathogen can survive in aerobic environment. Some authors have mentioned that aerotolerance is one of the survival mechanisms (Bronowski et al., 2014; Rodrigues et al., 2016) and other authors have recently reported that hyper-aerotolerant *C. jejuni* are highly prevalent in retail poultry meat (Oh et al., 2017; O`Kane and Connerton, 2017).

According to our results, *C. jejuni* was the most prevalent species in both, ambient and MAP products, representing 81.2 % of *Campylobacter* isolates. This data might be in relation with the high level of *C. jejuni* that cause infection in humans; that can reach 90 % of human infections (Skarp et al., 2016). Authors as Oh et al. (2015) found that the most hyper-aerotolerant *C. jejuni* strains belong to MLST 21 CC, which is the major genotype implicated in human gastroenteritis. Therefore, it is possible that *C. jejuni* belonging to this MLST with increased aerotolerance may survive well in foods and are more likely to reach humans; therefore cause human illnesses more frequently than aerosensitive *C. jejuni* strains (Oh et al., 2015; Oh et al., 2017).

Cross-contamination might have an important role, since it might increase *Campylobacter* prevalence. For instance, unpacked marinated products presented the highest *Campylobacter* contamination percentage (56.3 %) due to the manipulation in the butcher shops. Some authors suggested that polyphosphate used to marinate, enhance the survival of *Campylobacter* species in the exudate of treated poultry products (Gunther et al., 2015). Moreover, manipulation of raw poultry before ready-to-eat food, and do not wash the hands and/or the cutting board during handling of foods might be factors that increase cross-contamination (Signorini et al., 2013; Zbrun et al., 2017).

The prevalence of *Campylobacter* differed between chicken products; being packed thighs (with skin) the most contaminated products, as it has been described by other authors (Berrang et al., 2001; Chanarapanont et al., 2003; Davis and Conner, 2007 and Stella et al., 2017). On the contrary, minced products showed low *Campylobacter* prevalence compared to the rest of the fresh chicken products. This fact may be explained by the use of several additives including preservatives used in the manufacture of these products or by a higher exposition to environmental conditions. Similar results were obtained by Stella et al. (2017) in a recent study of retail poultry products in Italy.

Typing revealed a wide heterogenicity among isolates (204 isolates were clustered into 76 clusters). A similar genetic diversity has been reported by other authors (Di Giannatale et al., 2014; Pedonese et al., 2017). Moreover, our study suggests that some pulsotypes might be associated to the plant where samples come from. This is the case of pulsotypes j-37, j-44, j-54, where isolates with the same pulsotype were recovered during 58, 42 and 31 days respectively from different butcher shops, suggesting a possible common origin, as almost all products came from the main slaughterhouse of the city. Similar situation has been observed in cluster c-14, where strains of *C. coli* from the same product (thigh), from the same supplier (E2), were found in samples taken with a difference of 57 days. Moreover, in a previous study we found that some *C. jejuni* pulsotypes can persist in the poultry plant environment during at least 21 days (García-Sánchez et al., 2017). This

hypothesis is corroborated by other authors, who identified some *C. jejuni* profiles in the same processing plant, which re-appeared at different years, suggesting that some predominant PFGE patterns are associated to a given processing plant (Willians and Oyarzabal, 2012). Additionally, persistence might be observed as well inside the butcher shops. In cluster j-11 two strains, with the same pulsotype, were isolated from marinated products, in the same butcher shop in the space of two months. This fact does not exclude the possibility that some pulsotypes, which were found in the chicken products, came from the farms that supply the butcher shops or the processing plants, as it was described before by Melero et al. (2012).

Alternatively, some practices as freezing of meat, in both butcher shops and industries, may be taken in order to regulate the stock of fresh chicken products according to market demands. Freezing of *Campylobacter* positive broiler carcasses has been proposed as an intervention to reduce the incidence of *Campylobacter* in raw poultry products (Georgsson et al., 2006; Tustin et al., 2011). In that sense, Melero et al. (2013) found in chicken burgers, that freezing stress was an effective strategy to reduce *C. jejuni* counts; but only in combination with a high-O_2-MAP (50 % O_2:50 % CO_2) *Campylobacter* was completely eliminated.

The high prevalence of *Campylobacter* in fresh chicken retail products may be considered as a public health problem, since consumer might be exposed to this biological risk. Moreover, *Campylobacter* has developed resistance to several antimicrobial agents over the years, including (fluoro)quinolones and macrolides, which are the drugs of choice in treatments (Di Giannatale et al., 2014). In this study a high resistance in different genotypes has been observed, showing severe multi-drug resistant strains, especially within *C. coli* isolates. *C. jejuni* was mainly resistant to ciprofloxacin, nalidixic acid and tetracycline. Similar results have been described by other authors as Zhang et al. (2016) and Pedonese et al. (2017). Resistance of *Campylobacter* to (fluoro)quinolones was firstly reported in the late 1980s and since then, it has been increasing in many countries. An alarming situation is reported in this study, where the all C. *jejuni isolates* were resistant to this antibiotic.

Conclusions

This study provides information about the contamination levels and genetic diversity of *C. jejuni, C. coli* and *C. lari* in different fresh chicken products at retail in Spain. Results obtained suggest that some *Campylobacter* strains are more robust than previously thought, as they are able to survive in a broad range of different gas compositions and persist longer in the environment of the processing plant or butcher shop. Cross-contamination might play an important role in the high diversity of

Campylobacter strains found at retail level. However, it is still surprising that the same pulsotypes appear in different fresh chicken products coming from different suppliers and different geographical locations. This study confirms the increasing concern due to the rise in antibiotic resistance of *Campylobacter* spp. isolates in fresh chicken products at retail, which is the previous step before consumer. This fact suggests that a bigger effort must be done in previous steps to avoid or reduce the presence of this pathogen in those products. Moreover, consumer campaigns alerting about handling of fresh chicken products at home must be conducted. Further studies, will be done to find out common metabolic characteristics among those surviving strains to get insight in their adaptive mechanisms that allow them to persist along the food chain till retail.

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459 **Figure 1.** Geographical distribution of manufactured and fresh packed chicken products
460 suppliers in Spain sampled in this study.
461
462 **Figure 2.** Dendrogram of *C. jejuni* PFGE types
463
464 **Figure 3.** Dendrogram of *C. coli* and *C. lari* PFGE types.

464 Table 1: Presence of *Campylobacter* spp., pH and gas composition of packed chicken
465 products from different geographical origin and suppliers.

Supplier	Origin	Samples (n)	C+ (%)[a]	pH	O_2 (%)	CO_2 (%)
B1	West	8	37.5	5.9 ± 0.2	20.5 ± 0.4	53.9 ± 1.9
B2	East	12	58.3	5.9 ± 0.4	14.9 ± 4.0	53.5 ± 2.4
C	West	42	38.0	5.9 ± 0.5	70.0 ± 3.7	21.6 ± 1.9
D	North	17	52.9	6.0 ± 0.5	18.5 ± 2.1	2.6 ± 0.8
E1	North	22	45.5	6.1 ± 0.2	77.5 ± 2.7	17.7 ± 2.5
E2	Centre	18	94.4	6.0 ± 0.8	19.2 ± 0.9	2.1 ± 1.1
G[b]	Centre	21	14.3	6.1 ± 0.3	65.3 ± 1.6	21.3 ± 1.5
			19.1	6.2 ± 0.2	28.4 ± 3.3	11.0 ± 0.4
H	Centre	6	16.6	6.1 ± 0.1	71.5 ± 0.1	21.8 ± 0.0
I	Centre	26	46.2	5.9 ± 0.5	69.6 ± 1.7	20.6 ± 1.9
J	Centre	2	50.0	6.2 ± 0.1	19.8 ± 0.2	1.5 ± 0.1
L	East	32	9.4	6.5 ± 0.4	11.4 ± 0.3	23.4 ± 0.8

466 ([a]): *Campylobacter* spp. positive samples
467 ([b]): For this supplier, the upper row corresponds to thigh samples and the lower row to breast samples.

468 Table 2. Prevalence of the different species of *Campylobacter* in retail chicken products

Chicken product	***Campylobacter* spp**	***C. jejuni***	***C. coli***	***C. lari***	Mix
Unpacked					
Thigh	51.6 % (33/64)	96.9 % (32/33)	3.1 % (1/33)	-	-
Breast	51.6 % (33/64)	96.9 % (32/33)	3.1 % (1/33)	-	-
Minced	21.9 % (14/64)	85.7 % (12/14)	-	14.3 % (2/14)	-
Marinated	56.3 % (36/64)	69.4 % (25/36)	30.6 % (11/36)	-	-
All	**45.3 % (116/256)**	**87.1 % (101/116)**	**11.2 % (13/116)**	**1.7 % (2/116)**	-
Packed					
Thigh	56.3 % (36/64)	55.6 % (20/36)	30.6 % (11/36)	2.8 % (1/36)	11.0 %[a] (4/36[)]
Breast	45.3 % (29/64)	89.8 % (26/29)	3.4 % (1/29)	3.4 % (1/29)	3.4 %[a] (1/29)
Minced	14 % (9/64)	33.4 % (3/9)	44.4 % (4/9)	11.1 % (1/9)	11.1 %[b] (1/9)
Marinated	18.7 % (12/64)	58.4 % (7/12)	33.3 % (4/12)	-	8.3 %[a] (1/12)
All	**33.6 % (86/256)**	**65.1 % (56/86)**	**23.3 % (20/86)**	**3.4 % (3/86)**	**8.1 % (7/86)**

469 ([a]): Mix of *C.jejuni*/*C.coli*

470 ([b]): Mix of *C.jejuni*/*C.lari*

471 Table 3: Relation between different *Campylobacter* spp. pulsotypes and suppliers of
472 chicken products along sampling time.

PFGE type	Number of isolates	Chicken product[a]	Sampling time (days)	Suppliers/butcher shops[b]
C. jejuni				
j-8	11	b, t, m, n	21	a, b, c, d, g, h, i, C
j-10	5	b, t	44	C, I
j-11	3	b, m	58	j, d
j-13	3	b, t, m	16	g, e
j-23	5	b, t, m	59	a, b, f, i, I
j-24	9	b, t, m	95	B1, E2, c, j, h
j-27	6	b, t, m	14	d, f, g
j-29	3	b	1	G
j-30	7	b, t	72	B2, C, E1, E2
j-31	7	b, n	72	C, I, h
j-37	9	b, t, m, n	58	a, c, d, e, g, k
j-44	22	b, t, m, n	42	a, b, c, e, f, g, h, i
j-47	5	b, t, n	95	g, d, e, E
j-48	3	b, m, n	7	b
j-49	5	b, t, m, n	119	D, B1, h
j-54	11	b, t, m, n	31	a, b, c, d, e, f, g
C. coli				
c-1	2	m	1	D
c-7	2	m	7	k, l
c-9	3	t, b	1	E1
c-10	2	m	62	I, D
c-12	2	b, m	21	k, b
c-14	5	t	57	E2
c-16	6	t, b, m	105	C, E2, I, j
c-18	2	t	39	h, I
c-19	3	t	1	B1
C. lari				
l-2	4	t, b, n	25	E (North), c

473 ([a]): b: breast, t: tight; m: marinated, n: minced chicken products.
474 ([b]): from a-l: are different unpacked chicken products from butcher shops; from A-I: are different packed
475 chicken products from industrial suppliers

476 Table 4. Antimicrobial susceptibility and MIC in *C. jejuni* PFGE types.

PFGE Type	CIP	NAL	TC	ERY	AZT	GM
C. jejuni						
j-1	R (16) [a]	R (64) [a]	R (2)	S (0.50)	S (0.06)	S (1)
j-2	R (8)	R (128)	R (64)	S (0.50)	S (0.06)	S (1)
j-3	R (16)	R (128)	R (64)	S (0.50)	S (0.06)	S (1)
j-4	R (8)	R (64)	R (128)	S (1)	S (0.06)	S (0.50)
j-5	R (8)	R (64)	R (64)	S (0.50)	S (0.06)	S (0.50)
j-6	R (16)	R (64)	R (4)	S (0.50)	S (0.03)	S (0.50)
j-7	R (16)	R (128)	R (128)	S (1)	S (0.06)	S (0.50)
j-8	R (8)	R (128)	R (64)	S (0.50)	R (0.03)	S (0.50)
j-9	R (16)	R (128)	R (16)	S (1)	S (0.06)	S (1)
j-10	R (8)	R (128)	R (64)	S (0.25)	S (0.06)	S (1)
j-11	R (8-32) [b]	R (128)	R (64-128) [b]	S (0.50-1) [b]	S (0.03-0.06)[b]	S (0.50)
j-12	R (8)	R (128)	R (64)	S (0.50)	S (0.03)	S (1)
j-13	R (8)	R (128)	R (128)	S (0.50)	S (0.03)	S (0.50)
j-14	R (16)	R (128)	R (32)	S (0.50)	S (0.03)	S (0.50)
j-15	R (16)	R (128)	R (64)	S (1)	S (0.06)	S (1)
j-16	R (16)	R (128)	R (64)	S (1)	S (0.06)	S (1)
j-17	R (8)	R (128)	R (64)	S (0.25)	S (0.06)	S (1)
j-18	R (16)	R (128)	R (32)	S (1)	S (0.03)	S (1)
j-19	R (8)	R (128)	R (16)	S (0.50)	R (1)	S (1)
j-20	R (8)	R (128)	R (64)	S (0.50)	S (0.03)	S (1)
j-21	R (8)	R (128)	S (0.50)	S (0.50)	S (0.06)	S (0.50)
j-22	R (8)	R (128)	R (64)	S (0.50)	S (0.03)	S (1)
j-23	R (8-16) [b]	R (128)	R (8-128) [b]	S (0.50)	S (0.03)	S (0.50-1) [b]
j-24	R (8)	R (128)	R (128)	S (0.50)	S (0.06)	S (1)
j-25	R (8)	R (128)	R (8)	S (0.50)	S (0.06)	S (0.50)
j-26	R (8)	R (128)	R (8)	S (0.50)	S (0.06)	S (0.50)
j-27	R (8-16) [b]	R (128)	R (64-128) [b]	S (0.50-2) [b]	S (0.03-0.06)[b]	S (1)
j-28	R (16)	R (128)	R (32)	S (1)	S (0.03)	S (1)
j-29	R (8)	R (128)	R (128)	S (0.25)	S (0.06)	S (1)
j-30	R (8)	R (128)	R (64)	S (0.25)	S (0.06)	S (0.50)
j-31	R (8-16) [b]	R (128)	R (32-128)	S (0.5-2) [b]	S (0.03-0.06)[b]	S (0.5-1) [b]
j-32	R (16)	R (64)	R (4)	S (0.50)	S (0.03)	S (0.50)
j-33	R (8)	R (64)	R (128)	S (0.50)	S (0.03)	S (0.25)
j-34	R (16)	R (128)	R (64)	S (1)	S (0.12)	S (1)
j-35	R (8)	R (128)	R (64)	S (1)	S (0.06)	S (1)
j-36	R (8)	R (128)	R (32)	S (0.50)	S (0.03)	S (0.50)
j-37	R (8)	R (128)	R (128)	S (0.50)	S (0.06)	S (0.50)
j-38	R (8-16) [b]	R (128)	R (64-128)	S (1-2) [b]	S (0.06)	S (1)
j-39	R (16)	R (128)	R (128)	S (1)	S (0.06)	S (1)
j-40	R (8)	R (128)	R (64)	S (0.50)	S (0.03)	S (0.25)
j-41	R (8)	R (128)	R (16)	S (1)	S (0.06)	S (1)
j-42	R (8)	R (64)	R (64)	S (0.50)	S (0.03)	S (0.50)
j-43	R (8)	R (128)	R (64)	S (0.50)	S (0.03)	S (0.25)
j-44	R (8)	R (128)	R (64)	S (0.50)	S (0.03)	S (0.50)
j-45	R (16)	R (128)	R (64)	S (1)	S (0.06)	S (0.50)
j-46	R (8)	R (128)	R (64)	S (0.50)	S (0.03)	S (0.25)
j-47	R (8)	R (128)	R (64)	S (0.50)	S (0.03)	S (0.25)
j-48	R (8)	R (128)	R (64)	S (0.50)	S (0.03)	S (0.25)
j-49	R (8)	R (128)	R (64)	S (0.50)	S (0.03)	S (0.50)
j-50	R (16)	R (128)	R (128)	S (2)	S (0.06)	S (1)
j-51	R (8)	R (128)	R (64)	S (0.50)	S (0.03)	S (0.25)
j-52	R (32)	R (32)	R (16)	S (0.50)	S (0.03)	S (0.50)
j-53	R (32)	R (128)	R (32)	S (0.50)	S (0.06)	S (0.50)
j-54	R (8)	R (128)	R (64)	S (0.25)	S (0.03)	S (0.25)
j-55	R (8)	R (128)	R (64)	S (1)	S (0.06)	S (1)
Total	**100 % R (8-32)**	**100 % R (32-128)**	**98 % R (2-128)**	**100 % S (0.25-2)**	**96 % S (0.03-0.012)**	**100 % (0.25-1)**

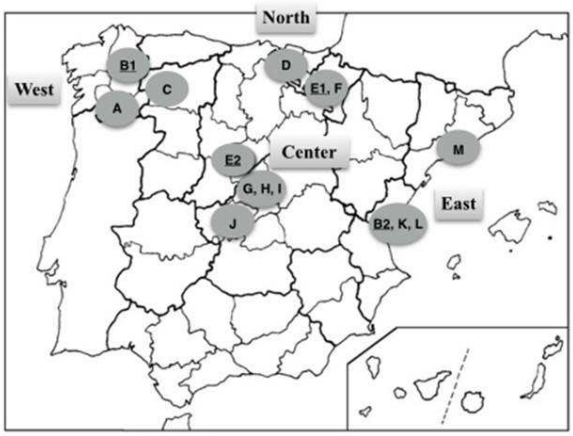
477 CIP: ciprofloxacin; NAL: nalidixic acid; TC: tetracycline; ERY: erythromycin; AZT: azithromycin; GM: gentamicin.
478 R= resistance; S=sensitive
479 ([a]): Interpretation of MIC for *Campylobacter* epidemiological cutoff values: NAL (16 mg/L); CIP (0.5 mg/L); TC (1 mg/L); ERY (4 mg/L);
480 AZT (0.25 mg/L) GM (2 mg/L).
481 ([b]): Isolates belonging to the same cluster with different MIC values.

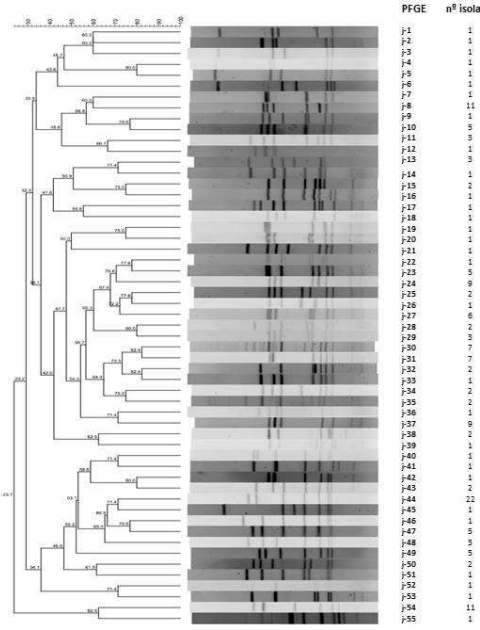
482 Table 5. Antimicrobial susceptibility and MIC in *C. coli* and *C. lari* PFGE types.

PFGE Type	CIP	NAL	TC	ERY	AZT	GM
C. coli						
c-1	R (16)	R (128) [a]	R (128)	S (1)	S (0.06)	S (1)
c-2	R (8)	R (128)	R (128)	R (16)	R (1)	S (1)
c-3	R (16)	R (128)	R (64)	R (16)	R (1)	S (0.50)
c-4	R (8)	R (128)	R (128)	R (16)	R (1)	S (1)
c-5	S (0.25)	R (16)	R (64)	S (1)	S (0.12)	S (1)
c-6	R (8)	R (128)	R (64)	S (0.50)	S (0.03)	S (1)
c-7	R (16)	R (128)	R (64)	S (0.25)	S (0.03)	S (1)
c-8	R (8)	R (64)	R (64)	R (16)	R (1)	S (1)
c-9	R (8-16) [b]	R (128)	R (64-128) [b]	R (16)	R (1)	S (1)
c-10	R (8-32) [b]	R (128)	R (128)	R (16)	R (1)	S (1)
c-11	R (16)	R (128)	R (128)	S (1)	S (0.12)	S (1)
c-12	R (8)	R (128)	R (64)	R (16)	R (1)	S (1)
c-13	S (0.25)	R (16)	R (64)	R (4)	R (0.25)	S (1)
c-14	S (0.25)	R (32)	R (32)	R (4)	R (0.25)	S (1)
c-15	R (16)	R (128)	R (32)	S (2)	S (0.06)	S (0.5)
c-16	R (16)	R (128)	R (128)	S (2)	S (0.12)	S (1)
c-17	R (16)	R (128)	R (128)	R (16)	R (1)	S (1)
c-18	S (0.25)	R (128)	R (32)	R (16)	R (1)	S (1)
c-19	R (16)	R (128)	R (128)	R (16)	R (1)	R (8)
Total	**79 % R (8-32)**	**100 % R (32-128)**	**100 % R (32-128)**	**63 % R (4-16)**	**63 % R (0.25-1)**	**95 % S (0.5-1)**
C. lari						
l-1	R (8)	R (128)	R (64)	S (1)	S (0.06)	R (2)
l-2	R (4)	R (128)	R (64)	S (0.50)	S (0.03)	S (0.25)

483 NAL: nalidixic acid; CIP: ciprofloxacin; TC: tetracycline; ERY: erythromycin; AZT: azithromycin: GM: gentamicin.
484 R= resistance; S=sensitive
485 ([a]): Interpretation of MIC for *Campylobacter* epidemiological cutoff values: NAL (16 mg/L); CIP (0.5 mg/L); TC (1 mg/L); ERY (4
486 mg/L); AZT (0.25 mg/L) Gm (2 mg/L).
487 ([b]): Isolates belonging to the some cluster presented MIC differences.

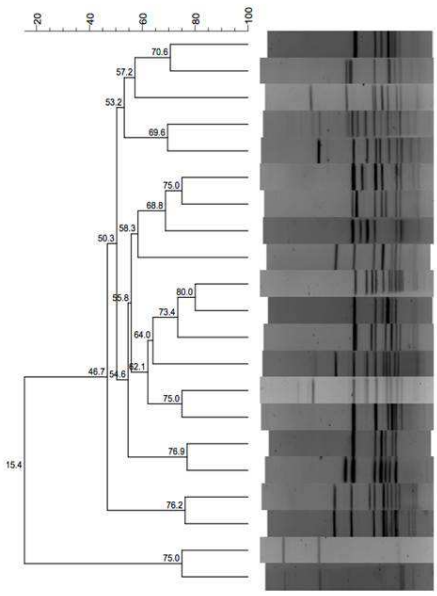
Figure 1





PFGE	nº isolates
j-1	1
j-2	1
j-3	1
j-4	1
j-5	1
j-6	1
j-7	1
j-8	11
j-9	1
j-10	3
j-11	3
j-12	1
j-13	3
j-14	1
j-15	2
j-16	1
j-17	1
j-18	1
j-19	1
j-20	1
j-21	1
j-22	1
j-23	5
j-24	9
j-25	2
j-26	1
j-27	6
j-28	2
j-29	3
j-30	7
j-31	7
j-32	2
j-33	1
j-34	2
j-35	2
j-36	1
j-37	9
j-38	2
j-39	1
j-40	1
j-41	1
j-42	1
j-43	2
j-44	22
j-45	1
j-46	1
j-47	5
j-48	3
j-49	5
j-50	2
j-51	1
j-52	1
j-53	1
j-54	11
j-55	1

Figure 2



PFGE	nº isolates
c-1	2
c-2	1
c-3	1
c-4	1
c-5	1
c-6	1
c-7	2
c-8	1
c-9	3
c-10	2
c-11	1
c-12	2
c-13	1
c-14	5
c-15	1
c-16	6
c-17	1
c-18	2
c-19	3
l-1	1
l-2	4

Figure 3

Highlights

High diversity of *C. jejuni* and *C. coli* was found among different fresh chicken products.

Campylobacter strains are able to survive in a broad range of gas compositions in MAP.

Some persistent PFGE types might play an important role in cross-contamination.

C. coli isolates showed the highest antimicrobial resistance.