Study of poultry livers as a source of *Campylobacter*

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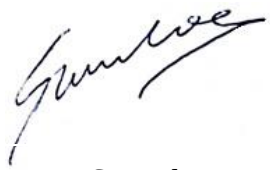

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Abstract

Campylobacter is the most common bacterial cause of human gastroenteritis in the world. The largest contributor of human *Campylobacter* infection in developed countries is considered poultry and poultry products. The current European legislation regulates levels of *Campylobacter* only on the neck skin from broiler carcases at a slaughterhouse. However, foodborne campylobacteriosis has also been linked to undercooked chicken liver.

There is no information on the occurrence of *Campylobacter* in chicken livers in Spain. Therefore, we decided to determine the prevalence and levels of *Campylobacter spp.* in chicken livers by sampling 56 flocks at slaughter in Catalonia, Spain. We studied 168 liver and ceca samples of three carcasses per flock collected randomly during the evisceration of the animals at two slaughterhouses. Overall *Campylobacter* prevalence was 54,76% in cecal samples, whilst in the liver surface and in the liver internal tissue was 71,43% and 35,12%, respectively. The data highlights chicken livers as a potential source of human campylobacteriosis, not only due to the *Campylobacter* prevalence but particularly because of the bacterial load, which was $>10^3$ CFU/liver or CFU/g in 40,12% of the samples of surface liver and in 6,59% of the internal tissue samples

1.Introduction

1.1*Campylobacter*

The genus *Campylobacter* consists of 47 species and belongs to the family *Campylobacteraceae*, under the order of *Campylobacterales*, in the class of *Epsilonproteobacteria* and in the phylum of proteobacteria (LPSN, 2020).

The genus *Campylobacter* are Gram-negative bacteria with microaerobic growth requirements. In morphological terms, campylobacters are usually S-shaped or spiral rods with tapering ends (0.2–0.8 μm-wide by 0.5–5 μm-long). The majority of the species have a corkscrew-like motion. This movement is possible due to a single polar flagellum at one or both ends of the cell. Flagella and flagellar motility are vital to many aspects of *Campylobacter* biology, including host colonization, virulence in ferret models, secretion and host-cell invasion (Chlebicz and Slizewska, 2018; Silva *et al.*, 2011; Young *et al.*, 2007).

The majority of *Campylobacter spp.* are microaerophilic and need reduced oxygen (3-10%) and raised CO_2 (5-10%) levels (Nachamkin *et al.*, 2008). Most *Campylobacter* species have a respiratory type of metabolism; however, several species (*Campylobacter concisus, Campylobacter curvus, Campylobacter rectus, Campylobacter mucosalis, Campylobacter showae, Campylobacter gracilis*, and, to a certain extent, *Campylobacter hyointestinalis*) require hydrogen or formate as an electron donor for microaerobic growth (Kaakoush *et al.*, 2015). For *Campylobacter* the optimum value for growth of water activity (aw) is 0,997 and a pH of 6.5–7.5. (Chlebicz and Slizewska, 2018; Silva *et al.*, 2011)

Campylobacter spp. grow at temperatures between 37°C and 42°C, with thermophilic species having an optimal growth temperature of 42°C (Nachamkin *et al.*, 2008). Thermophilic *Campylobacter* are the species causing gastrointestinal disease. The most frequent are *C. jejuni* and *C. coli* (Chlebicz and Slizewska, 2018; WHO, 2018), while *C. lari* and *C. upsaliensis* have also been isolated from patients with diarrhoeal disease but are reported less frequently (WHO, 2018).

1.2 Campylobacteriosis

Campylobacter is the most common bacterial cause of human gastroenteritis in the world and campylobacteriosis is the most reported zoonotic disease in the EU (EFSA and ECDC, 2019). According to Spanish National Epidemiological Surveillance Network (RENAVE), between 2014 and 2017 there was an increase in total number of notified cases of campylobacteriosis in Spain, with 122 outbreaks being notified and 536 people affected (RENAVE, 2020). Currently, there is no national or European control programme at the farm level which is probably because of the incomplete knowledge of the epidemiology of *Campylobacter* (Sevilla-Navarro *et al.*,2020, Urdaneta, 2016).

There has been a rise in the global incidence of campylobacteriosis in the past decade. The numbers of cases of campylobacteriosis have increased in North America, Europe, and Australia. This rise may be a consequence of many factors, including problems in detection and failure to effectively prevent transmission (Kaakoush *et al.,* 2015). Furthermore, the reported cases of *C. jejuni* and *C. coli* infections may only represent the tip of the iceberg due to underreporting (Wagenaar *et al.,* 2013). The ability of *C. jejuni* and *C. coli* to colonize and survive in a wide variety of animal species and habitats make them extremely difficult to control (Epps *et al.,* 2013).

The outcome of the disease depends on many factors. In most of the cases, the disease is self-limited, and the patient recovers within a few days (WHO, 2018). However, different patterns in the manifestation of the disease exist in developed and developing countries. In the developed world, campylobacteriosis manifests as bloody diarrhoea with mucus, and is usually self-limiting. In the developing world, watery diarrhoea predominates, and infection is more frequent among children, what makes them develop immunity as adults (Young *et al.,* 2007). The clinical features of *Campylobacter* enteritis due to *C. jejuni* and *C. coli* are clinically indistinguishable from each other and from illnesses caused by other bacterial enteric pathogens. That is why it is not possible to diagnose campylobacteriosis on the basis of symptoms alone and the stool culture is needed. Apart from that, *Campylobacter spp.* have been associated with a range of gastrointestinal conditions. There are two major late onset complications of *Campylobacter* infection: reactive arthritis and Guillain-Barré syndrome (GBS) (Allos, 2019). Generally, antimicrobial therapy is not indicated, except in severe cases and for immunocompromised patients (WHO, 2018).

1.3Transmission and reservoirs of *Campylobacter*

Campylobacter spp. are widely distributed in most warm-blooded animals. They are prevalent in domestic livestock, such as poultry, cattle, pigs, and sheep, as well as in a wide variety of wildlife (Kaakoush *et al.,* 2015). The bacteria have also been found in shellfish (WHO, 2018).

The main route of transmission of *Campylobacter* is generally believed to be foodborne, via undercooked meat and meat products, as well as raw or contaminated milk. Contaminated water or ice is also a source of infection (WHO, 2018). *Campylobacter* infection in developing countries is mainly caused by environmental and food contamination whereas, in developed countries, the primary source of *Campylobacter* are food production animals. The largest contributor of human *Campylobacter* infection in developed countries is considered poultry and poultry products (Kaakoush *et al.,* 2015). Broiler flocks are a natural host for *Campylobacter spp.* and colonized birds may carry a very high *Campylobacter* load in their gastrointestinal tract (Powell *et al*., 2012) but remain mostly asymptomatic intestinal carriers (Young *et al.,* 2007). *C. jejuni* colonizes primary the deep crypts of the caecum, where it is found in the mucus layer close to the epithelial cells (Young *et al.,* 2007).

If broiler houses are adequately cleaned and disinfected prior to arrival of the new animals, the flocks usually stay free of *Campylobacter* in the first 1–2 weeks. Once introduced into a flock, *Campylobacter* spreads rapidly an most of the animals become colonized, shedding up to 10^8 *Campylobacter*/g of cecal contents. These counts remain at a similar level till slaughter (Wagenaar *et al.,* 2013).

Studies, demonstrate that *Campylobacter* has a seasonal pattern, both in broiler flocks and in human infections, with highest rates seen during warmer months (Jore *et al*.,2010; Nylen *et al.*,2002). However, it is still unclear how seasonality and temperature may affect *Campylobacter* colonization of broilers. (Jorgensen *et al.*,2011; Lawes *et al*., 2012)

1.4 *Campylobacter* regulations.

In 2018, came into force the European Regulation 2017/1495 on *Campylobacter* in broiler carcases. Under this regulation neck skin from broiler carcases at a slaughterhouse must be analysed for *Campylobacter* after chilling, with the microbiological content at a

maximum of 1000cfu/g (colony-forming units per gram) (European Commission, 2017). A recent study performed in Spain suggest that the prevalence of *Campylobacter* in broiler carcases that exceed the 1000cfu/g limit is low (Sevilla-Navarro *et al.*,2020). However, it is important to remember that direct comparison of results should be performed with caution due to differences in experimental designs of studies and countries.

1.5 *Campylobacter* in the liver

C. jejuni mainly colonizes poultry and is found predominantly in the cecum and colon but is also present in the liver (Epps *et al.*, 2013; Berrang *et al.*, 2019). Despite it has long been known that *Campylobacter* can be found in chicken livers, (Barot *et al.*, 1983) still little information is available regarding the risk posed to the consumer by their handling and consumption. A study performed in Scotland showed that prevalence of *Campylobacter spp.* in retail chicken livers was 81% (Strachan *et al.*, 2011). This clearly indicates significant contamination across chicken livers and confirms its important role of this food as a potential source of infection in humans. The cooking trends of the livers in restaurants pose a risk of infection. A study performed in 2015 in the United Kingdom showed that most chefs correctly identify safely cooked livers, but they prefer to serve them slightly raw. Also, it was estimated that 19%–52% of livers served commercially in the United Kingdom fail to reach 70°C and in this case *Campylobacter* survival rates are 48%–98% (Jones *et al.*, 2016). Also, there have been multiple outbreaks of campylobacteriosis attributed to undercooked or mishandled chicken livers all over the world (Edwards *et al.*, 2013; Lahti *et al*., 2017; Lanier *et al.*, 2018; Little *et al*., 2010; Parry *et al*.,2012). These outbreaks may be caused by two main factors: inadequate cooking and pathogen contamination. Safe handling of raw meat and other raw food ingredients, thorough cooking and good kitchen hygiene can prevent or reduce the risk posed by contaminated food (EFSA and ECDC, 2019).

2. Objective

There is no information on the occurrence of *Campylobacter* in chicken livers in Spain. Thus, the aim of this study was to determine the prevalence and levels of *C. jejuni* and *C. coli* in chicken livers from two slaughterhouses in Catalonia, one of the main poultry producers in Spain.

3. Material and methods

3.1. Sample collection and preparation

Sampling was performed in two different slaughterhouses from Catalonia between October 2019 and September 2020.

Liver and ceca samples of three carcasses per flock were collected randomly during the evisceration of the animals in the slaughterhouses. Ceca and liver were placed separately into new, clean plastic bags. Samples were transported refrigerated to the laboratory, where they were processed within 24h.

Ceca were removed from the bags and placed on Petri dishes. They were opened with sterile scissors. Contents of the two ceca were homogenized with a swab and streaked onto Charcoal Cefoperozone Deoxycholate agar plates (CCDA, CM739 with selective supplement, SR0155E; Oxoid, Basingstoke, UK). Plates were incubated for 48 h at 42ºC in microaerophilic conditions using a microaerobic atmosphere generator (Campygen, CN0025A, Oxoid, Basingstoke, UK). We then subcultured two presumptive colonies per positive sample onto blood agar plates (BioMerieux, Marcy l'Etoile, France) at 37ºC during 48h in microaerobic conditions.

Campylobacter detection and quantification from livers was attempted from the surface and from the inner tissue. To sample the surface of the livers, 50 ml of buffered peptone water (BPW, Oxoid, Basingstoke, UK) was introduced into the plastic bags containing the livers, massaged for 2 minutes and let to settle down for 5 minutes. Qualitative detection was performed both by direct plating and with preenrichment in Bolton broth (CM0983, with selective supplement SR0183E and laked horse blood, SR0048C; Oxoid, Basingstoke, UK). For direct plating and quantitative detection of *Campylobacter*, 100 µl and ten-fold dilutions of the homogenate were streaked onto CCDA plates in duplicate, and incubated as described above. When performing the preenrichment step, 10 ml of the homogenate was mixed with 10 ml of 2x Bolton broth. Preenrichment cultures were incubated at 37ºC for 4h followed by 44h at 42ºC under microaerobic conditions. Next, 100 µl of the cultures were inoculated onto CCDA plates and incubated for 48-72h at 42 ºC under microaerobic conditions.

To sample internal tissue, livers were placed on a sterile Petri dish and were sanitized by searing of the surface with a hot spatula. With sterile scissors and tweezers 2 g of internal tissue was obtained. A portion of 1 g was mixed with 5 ml of BPW, and 100 µl and a ten-fold dilution were platted onto CCDA in duplicate, as above. The remaining 1 g was preenriched in 3 ml of Bolton broth and 100µl was inoculated onto CCDA. Plates were incubated for 48-72h at 42 ºC under microaerobic conditions.

For *Campylobacter* quantification, colony counts on CCDA was performed and when needed, *Campylobacter* colonies were confirmed using an agglutination test (Dryspot, DR0155, Oxoid, Basingstoke, UK).

Up to four presumptive colonies on CCDA plates were subcultured onto blood agar plates (both from the surface and inner tissue of the liver).

Isolates were preserved in brain heart infusion broth with 20% glycerol at -80 ºC.

3.2 Identification of *Campylobacter* species

Identification of the isolates at the species level was performed by multiplex PCR using primers targeting the lipid A gene lpxA (Klena *et al*., 2004) with forward primers lpxA-*C. coli* 5'- AGACAAATAAGAGAGAATCAG-3' and lpxA-*C. jejuni* 5'-ACAACTTGGTGACGATGTTGTA-3', and a reverse primer lpxA-RKK2m 5'-CAATCATGDGCDATATGASAATAHGCCAT-3' for both *C. coli* and *C. jejuni*. DNA extraction was performed from a bacterial suspension in PBS using InstaGene Matrix (Bio-Rad Laboratories, Hercules, CA, USA) following the manufacturer instructions.

PCR amplification was performed in 25 µl containing 2.5 µl of DNA, 12.5 µl of a PCR master mix (M7502, Promega Corporation, Madison, WI, USA), 2.5 µl of a 1 µg/µl BSA solution, 10 pmol of each forward primer and 20 pmol of reverse primer lpxA-RKK2m and 3.5 µl of nuclease-free water. DNA amplification was performed in a Thermal Cycler (Gene Amp PCR System 9700, Applied Biosystems, Singapore) and the conditions were: 1 cycle at 94°C for 5 min, followed by 30 cycles at 94°C for 1 min, 50°C for 1 min and 72°C for 1 min; the last elongation step at 72°C lasted 7 min. Amplicons were detected by gel electrophoresis using 1.8% agarose gels containing 0.2 µg/ml of ethidium bromide. As a reference a DNA molecular weight marker 2 log DNA ladder (New England Biolabs, Ipswich, MA, USA) was included.

3.3 Statistical Analysis

Prevalence of *Campylobacter* from liver surface, inner tissue, and cecal contents were compared by chi square test for independence. Significance was assigned at $P \leq 0.05$.

4. Results and Discussion

The study included samples of 56 batches, from which three carcasses per batch were analysed. The total number of ceca and livers sampled was 168 each.

Of the 168 chicken ceca samples tested, 54,76% were positive for *Campylobacter*. This prevalence is similar to what has been previously reported on average in north-eastern Spain (Urdaneta, 2016). With regards to liver samples, 71,43% were positive for *Campylobacter* on the outside surface and 35,12% in the internal tissue. High prevalence of *Campylobacter* on the outside surface of the liver was also shown in other studies (Berrang *et al.*, 2019; Simaluiza *et al.*, 2015; Berrang *et al.*, 2018), with some being even 100% (Whyte *et al.*, 2006). The prevalence of *Campylobacter* in the internal tissue of the liver, was higher than 31% reported in the study from USA (Berrang *et al.*, 2018) and lower than 90% reported in a study from New Zealand (Whyte *et al.*, 2006).

Table 1. *Campylobacter* occurrence in broiler carcasses according to sample type (n = 168).

Ceca	Outer liver	Inner liver	Number of pos. samples (%)
+	+	+	30,36% (51/168)
+	–	–	4,76% (8/168)
+	–	+	1,79% (3/168)
+	+	–	17,86% (30/168)
–	+	+	2,38% (4/168)
–	+	–	20,83% (35/168)
–	–	+	0,60 (1/168)
–	–	–	21,43 (36/168)

As shown in table 1, the majority of the samples (30,36%) were positive in all processed samples per carcass (ceca and liver, both external surfaces and internal tissue), which suggests: a) cross-contamination from the ceca to the external surface of the livers, which needs further analysis to confirm by genotyping isolates from both kind of samples: and b) an internal migration of *Campylobacter* to extraintestinal sites, such as the liver. This would support results obtained in other studies. Some suggest that there is possible internal migration of *C. jejuni* between the gall bladder, the bile duct and the liver (Moore and Madden, 1998; Garcia *et al.*, 1985). However, in this study we did not determine presence of *Campylobacter* in gall bladder nor bile duct. Therefore, further study on this

topic is needed to clarify this aspect. Also, a proportion of carcasses (17,86%) were only positive for ceca and the external liver samples, pointing also to a cross-contamination during processing.

On the contrary, a small proportion of carcasses were *Campylobacter*-positive only for the ceca, but not for any liver sample (4,76%), which suggests that few carcasses undergo neither cross-contamination of the liver through the ceca nor extraintestinal *Campylobacter* migration. Similarly, few carcasses (1,79%) were *Campylobacter* negative for the external surface of the livers while positive both for the ceca and the internal liver tissue.

However, in some cases, ceca were *Campylobacter*-negative, while livers were positive (either internal or external samples, or both). There are two possible explanations for such outcome. It is possible that ceca were positive but we did not detect it, probably due to an overgrowth of accompanying microbiota which has masked *Campylobacter*, or there was a cross contamination of the livers during processing at the slaughterhouses. The striking relatively high prevalence (20,83%) of *Campylobacter*-positive samples of liver surface, while the cecal and inner liver tissue were negative, suggests cross-contamination between batches during processing at the slaughterhouses.

Table 2. *Campylobacter* occurrence in broiler batches according to sample type (n = 56).

Ceca	Outer liver	Inner liver	Number of pos. flocks (%)
+	+	+	26 (46,43%)
+	–	–	1 (1,79%)
+	–	+	0
+	+	–	6 (10,71%)
–	+	+	2 (3,57%)
–	+	–	14 (25,00%)
–	–	+	1 (1,79%)
–	–	–	6 (10,71%)

For each batch 3 carcasses were analysed. A batch was considered positive if at least one sample was positive

For each kind of sample, a batch was considered positive if at least one sample from each of the three processed carcasses per batch was positive. In total, 89,29% (50/56) of the batches were positive for *Campylobacter* in at least one of three samples. For 26 of 56 batches (46,43%), *Campylobacter* was detected in all samples (liver surface, internal liver

tissue, and ceca), while only 10,71% of the batches were negative for all three kind of samples and as little as 1,79% of batches were liver negative while the ceca were positive. The 25% of batches that were *Campylobacter*-positive both for cecal and external liver samples suggests a cross-contamination of the liver during processing, as indicated above.

Table 3. *Campylobacter* load in chicken livers.

CFU/U	**Percentage (number of samples/total number of samples)**	
	External Count (CFU/liver)	**Internal Count (CFU/g)**
>1000	40,12% (67/167)	6,59% (11/167)
500-1000	6,59% (11/167)	1,20% (2/167)
<500	53,29% (89/167)	92,22% (154/167)

Table 3 shows the *Campylobacter* load of broiler livers, both for external surface of whole liver and for internal tissue. The European Regulation 2017/1495 establishes that the skin of the neck from broiler carcases at a slaughterhouse cannot exceed the maximum *Campylobacter* content of 1000cfu/g (European Commission, 2017). When extrapolating this limit with the bacterial load found in the internal liver samples, most of the samples are well below the 1000 CFU/g. However, a high proportion (40,12%) of whole livers are above this limit. Taking into account that the infectious dose for *Campylobacter* is around 1000 UFC/ml and that it can be as low as 500 UFC/ml (Robinson, 1981; Black *et al.*,1988), our results points to a health risk when consuming livers, even if care is taken to avoid cross-contamination at the kitchen. A study showed that livers can be free of *Campylobacter* after cooking (Whyte *et al.*, 2006), but the cooking trends to serve chicken livers slightly raw (Jones *et al.*, 2016), constitutes a risk of infection.

However, it should be noted that 35,44% of negative samples when performing enumeration, were positive after enrichment. This shows the importance to perform this enrichment step to increase the chances to detect *Campylobacter* in livers, but also that a relevant number of livers despite carrying *Campylobacter*, the bacterial load is rather low, thus posing a low risk of infection to consumers.

Table 4. *Campylobacter* species in chicken samples.

Campylobacter species	**Prevalence (positive/total)**		
	Cecum	**External liver**	**Internal liver**
C. coli	45,65% (42/92)	40% (48/120)	37,29% (22/59)
C. jejuni	48,91% (45/92)	45,83% (55/120)	52,54% (31/59)
C. coli and *C. jejuni*	5,43% (5/92)	14,17% (17/120)	10,17% (6/59)

The majority of the samples were identified as *C. jejuni*. Of the 120 isolates of external surface of the liver identified, the majority were *C. jejuni*. This is in line with the results of other studies made in Ecuador (Simaluiza *et al.*, 2015), United States of America (Noormohamed, and Fakhr, 2012) and New Zealand (Whyte *et al.*, 2006), but a bit different to previous cross-sectional studies performed at CReSA analysing cecal samples (Urdaneta, 2016). Furthermore, as in the previous studies (Berrang *et al.*, 2019; Berrang *et al.*, 2018), the majority of the internal tissue of the liver samples, were identified as *C.jejuni*. However, there is no relation between *Campylobacter* species (*C. jejuni*/*C. coli*) identified and type of the sample (P> 0,05).

Coinfections (*C. jejuni* and *C. coli*) were more prevalent in the livers than in the ceca. This may be due to: cross-contamination of the livers in the slaughterhouses and/or not detecting coinfections in the samples of the ceca.

Table 5. *Campylobacter*-positive samples according to the season.

Season	**Prevalence (positive / total in each season)**		
	Cecum	**External liver**	**Internal liver**
autumn - winter	40,74% (22/54)	74,07% (40/54)	31,48% (17/54)
spring - summer	61,40% (70/114)	70,18% (80/114)	36,84% (42/114)

It is well known that *Campylobacter* shows seasonality, although it is usually more marked in northern countries compared with temperate ones (Jore *et al.*,2010; Jorgensen *et al.*,2011). In this study, only *Campylobacter* in the ceca was detected significantly more often during spring-summer period (P<0,05).

The mechanism by which seasonality/temperature affects *Campylobacter* colonization of broilers is unclear. It may be linked to changes in flock management, increased

load/survival of *Campylobacter* organisms in the environment or to greater impact of wildlife vectors associated with higher temperature. (Jorgensen *et al.*,2011; Lawes *et al.*, 2012; Urdaneta, 2016).

5. Conclusions

- Chicken livers are often contaminated by *Campylobacter*, both externally and internally.
- Chicken livers represent a potential risk of human campylobacteriosis, both because of the prevalence of positive livers and because of the bacterial load.
- Results of this study suggests a frequent cross-contamination of livers between batches during processing at the slaughterhouses.
- The most frequent species isolated from all kind of samples was *C. jejuni* followed by *C. coli*.
- Results of the study confirms a seasonal pattern of *Campylobacter* in broilers with higher prevalence during warmer months.
- Further investigation is required to determine the potential risk of campylobacteriosis due to consumption of chicken livers. and the relevance of the internal migration of *Campylobacter* to extraintestinal sites.
- More research is needed to test practical and immediately applicable methods of chicken liver decontamination.

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