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In a warm climate, ventilation, indoor temperature and outdoor relative humidity have significant effects on *Campylobacter* spp. colonization in chicken broiler farms which can occur in only 2 days

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Abstract

A longitudinal study was conducted in five conventional broiler farms during a 2-year period to determine the dynamics of *Campylobacter* infection in a warm climate region (north-eastern Spain). Weekly sampling of 63 flocks was performed upon one-day-old chick placement, including animal and environmental samples. *Campylobacter* spp. detection was assessed by culture and non-culture methods. Environmental samples were also obtained from cleaned and disinfected houses prior to chick placement. Thirty-nine flocks (61.90%) became colonized during the growing period. First bird excreting *Campylobacter* was detected in 10-day-old chicks and the earliest a whole flock became positive was at 14 days of age, while the latest was at 39 days. Once *Campylobacter* was detected in chickens, the whole flock was colonized within 2 to 13 days. *Campylobacter* farm prevalence (positive flocks) ranged from 53.85% to 83.33% in four out of five farms, while the remaining farm showed a lower prevalence (38.5%). Logistic regression model showed that *Campylobacter* infection was more likely under higher minimal indoor temperature as well as at higher minimal outdoor relative humidity, characteristic of warm climates such as those from Mediterranean countries. Ventilation type was also significant (P= 0.021). No clear farm effect or seasonality were observed. Biosecurity improvements, specially at house level, are needed in broiler farms to prevent flock colonization and reduce the current high flock prevalence.

Keywords: colonization dynamics, poultry farms, boot socks, intensive sampling, environment, housed chickens

1. Introduction

Campylobacter spp. is a major cause of foodborne diarrhoeal illness in humans worldwide (Jacobs-Reitsma, 2000; Humphrey, 2006; Kaakoush *et al*., 2015). In the European Union, campylobacteriosis remains the most commonly reported foodborne disease in humans since 2005, and in 2020 it represented more than 60% of all the reported zoonosis cases (EFSA and ECDC, 2021). Many more cases go undiagnosed or unreported and the true incidence is estimated in 9 million cases per year (EFSA, 2011). The high incidence of *Campylobacter* diarrhoea, as well as its duration and possible sequelae, makes it highly important from a socio-economic perspective (Havelaar *et al*., 2015). *Campylobacter jejuni* is the main species identified and the most common predisposing factor to the peripheral neuropathies Guillain-Barré and Miller-Fisher syndromes (Winer, 2001; Leonard *et al*., 2004).

Campylobacter spp. is present in the intestinal tract of all types of domestic livestock and many wild animals (Humphrey *et al*., 2007). However, poultry meat is considered the primary source of human infection, due to an improper handling, preparation or consumption of contaminated meat (EFSA and ECDC, 2021). The prevalence of *Campylobacter* spp. in broiler chicken batches varies considerably between EU countries. In 2018, prevalence ranged from 3.50% to 100% (Spain, 59.78%; EU average 25.96%) (EFSA and ECDC, 2019). Also, since not only many chickens are *Campylobacter*-positive, but also contamination levels can be extremely high, there is an urgent need to reduce both the prevalence and the levels of carcass contamination to reduce the risk of infection in humans. In 2020, EFSA experts reviewed on-farm control options for *Campylobacter* in broilers and estimated that a 3-$\log_{10}$ reduction in broiler caecal concentrations would reduce the relative EU risk of human campylobacteriosis attributable to broiler meat by 58%, rather than the former estimates of over 90% from 2011 (EFSA BIOHAZ Panel, 2020).

On-farm control measures to block the initial colonization are likely to be the most cost-effective (van Wagenberg *et al*., 2016; Sibanda *et al*., 2018). This is because intestinal contamination is the main source of broiler carcass contamination at the slaughterhouse, since campylobacters accumulate to high numbers in chickens, and may persist in the gut until slaughter age, increasing the subsequent spread during slaughter (Melero *et al*., 2012; EFSA and ECDC, 2013; Sevilla-Navarro *et al*., 2020). Thus, *Campylobacter* load is likely to increase along the different stages of the production chain (Mota-Gutierrez *et al*., 2022). However, to implement an effective control on farm, it is important to identify the sources and routes of infection in housed flocks. This is particularly relevant in the warmer regions, such as southern Europe, where a higher *Campylobacter* flock prevalence occurs throughout the year, compared to the colder northern countries, with a more marked seasonality (Sommer *et al*, 2016). Also, there is scarce information regarding the epidemiology and dynamics of infection of *Campylobacter* on farm in southern Europe, compared to northern countries (Wingstrand *et al*., 2006; Hofshagen and Kruse, 2005; Hald *et al*., 2008). Different farming practices may also influence *Campylobacter* infection of broiler flocks, as may climatic conditions (McDowell *et al*., 2008; Rushton *et al*., 2009; Kittler *et al*., 2021).

Thus, with the aim of better understanding the epidemiology of *Campylobacter* in southern Europe, as well as to gain insight into the colonization dynamics and the influence of environmental factors in the infection and transmission of *Campylobacter* spp., a two-year longitudinal study was carried out in five Spanish broiler farms

2. Material and methods

2.1. Study design

Five broiler farms, each belonging to a different broiler company in Catalonia (northeastern Spain), where selected for a two-year longitudinal study. All broiler companies are

the major producers in Catalonia and one of them is also the main broiler company in Spain. All of them had a breed, integrated system. Characteristics of the farms are depicted in Table 1.

From June 2011 to October 2013, the longitudinal monitoring of all flocks produced in all five farms was performed by sampling both the birds and the external and internal environment of the houses. In those farms with more than one broiler house (two-broiler house farms), the intensive sampling was performed in one of the houses (study house), with additional boot sock sampling of the second house (neighbour house).

2.2. Boot socks and one-day old chicks sampling

Boot socks sampling was performed for the early detection of *Campylobacter* infection. Boot socks were previously moistened in sterile saline solution and worn over plastic overboots. Sampling was performed separately both outside (path leading to the study house) and inside the studied broiler houses (anteroom and broiler room), as well as inside the additional broiler houses (broiler room of the neighbour house) in the two-house farms. Inside each broiler house, boot sock sampling was performed by walking twice the whole broiler room at day 0 (cleaned and disinfected broiler houses prior to chick placement), upon one-day-old chick placement, and weekly from day 7 (seven-day-old chicks) until slaughter or until a boot sock was positive. At day 0, 1 and 7 sampling was performed with one pair of boot socks; from day 14 onwards, weekly samplings included 6 pairs of boot sock samples, one from each of the six areas covering the whole broiler floor in the monitored broiler house. Samples were transported in sterile sealed zip bags to the laboratory in a cool box and processed on the same day of collection.

Also, in order to confirm that day-old chicks were free of *Campylobacter* upon placement, three random samples of lining paper soiled with faeces from crates in which

chicks were transported were sent refrigerated to the laboratory for PCR detection of *Campylobacter* spp.

Animal studies were performed in accordance with the regulations required by the Ethics Commission in Animal Experimentation of the Generalitat de Catalunya (Approved Protocol number 4239).

2.3. Cloacal swabs sampling

Once a boot sock sample was positive at the broiler room from the study house, cloacal swab samples were obtained from 30 live birds in each study house, by sampling 5 birds from six different areas covering the whole broiler room. Samples were collected at least every 7 days, and upon first *Campylobacter* isolation, the sampling frequency increased to every 2-4 days until all 30 cloacal swabs were positive. At that point, the flock was considered positive and sampling finished. Negative flocks were sampled weekly until slaughter, with a maximum of 9 samplings per flock.

Cloacal swabs were transported in Amies with charcoal medium (Deltalab, Barcelona, Spain) and processed in the laboratory individually, as described below.

2.4. Feed, water and litter samples

Feed samples (~400g) were collected at each weekly visit to the farms from the automatic broiler troughs inside the broiler houses and further processed for *Campylobacter* detection as described below.

Samples of 300 ml of water were collected from the water tank in the anteroom and filtered through 0.45 μm membranes (Millipore, Darmstadt, Germany). Filters were then aseptically transferred to 100 ml of Bolton broth (CM0983 with selective supplement SR0183;

Oxoid, Basingstoke, UK) in containers with a small headspace and tightly closed lids and incubated at 37ºC for 4h and at 42ºC for 20h. After enrichment, 100 µl of the enrichment broth was streaked onto mCCDA agar plates and incubated at 42ºC for 48h in a microaerobic atmosphere (8-10 % v/v $C0_2$ and 5-7 % O_2; Anaerocult C; Merck, Darmstadt, Germany).

Composite samples of litter from six areas of the study house were collected during the weekly visits to farms, placed in a zip bag and carefully sealed, for pH and moisture measurements.

2.5. Campylobacter detection

2.5.1. DNA extraction

A 200 ml of 0.85% sterile saline solution was added to the zip bag containing each pair of boot sock samples (10 g) and the bag was hand palpated for 1 min to release faecal material from the fabric. The homogenate was let to settle for 10 minutes, and then 1 ml of the supernatant was collected and centrifuged in an Eppendorf 5415D equipment (Eppendorf, Spain) at 16,100 g for 7 min. The supernatant was discarded, and the pellet was processed for DNA extraction using the Wizard® Genomic DNA Purification Kit (Promega Corporation, Madison, USA), according to manufacturer recommendations.

Composite samples of 10 g from day-old chicks lining paper soiled with faeces, from crates in which chicks were transported were homogenized in 200 ml sterile saline solution and DNA extraction was performed as for boot sock samples.

DNA extraction from feed samples was performed similarly to boot sock samples. A 12 g of feed was weighed, suspended in 200 ml of sterile saline solution and 1 ml of the homogenate was used for DNA extraction.

2.5.2. PCR

Campylobacter detection by PCR was performed by using the primer pairs C412F (5`-GGATGACACTTTTCGGAGC-3`) and Camp R2 (5`-GGC TTC ATG CTC TCG AGT T-3'), as described previously (Katzav *et al*., 2008). These primers are based on the 16S rDNA, which generates an amplicon of 857 bp. Briefly, PCR amplification was performed in 25 µl containing 2.5 µl of DNA suspension, 12.5µl of a PCR master mix (Ref. M7502, Promega Corporation, Madison, USA), 2.5µl of BSA (1µg/µl) and 1µl (10 pmols/µl) of each forward and reverse primers, and 5.5µl nuclease fee water. The amplification was performed in a Thermal Cycler (GeneAmp PCR System 9700, Applied Biosystems, Singapore), and the conditions were: one cycle at 94ºC for 5 min, followed by 30 cycles at 94ºC for 1 min, 58ºC for 1 min and 72ºC for 1 min, with a final extension at 72ºC for 7 min.

2.6. Campylobacter isolation and identification

Once any boot sock sample from the study house was *Campylobacter* positive (by means of PCR detection), cloacal swabs were individually streaked onto *Campylobacter* blood-free selective agar (mCCDA, modified charcoal cefoperazone deoxycholate agar, CM739 with selective supplement, SR0155E; Oxoid, Basingstoke, UK) and incubated at 42 °C for 48 h in a microaerobic atmosphere (Anaerocult C; Merck, Darmstadt, Germany), for *Campylobacter* isolation and identification as previously described (Urdaneta *et al*., 2015). If different colony morphologies were found on a mCCDA plate, at least one of each was picked and subcultured onto blood agar plates (Biomerieux, France). Presumptive *Campylobacter* isolates were confirmed and identified at species level by PCR with primer pairs specific for *C. jejuni* and *C. coli* (Klena *et al*., 2004).

2.7. Environmental measurements

2.7.1. Litter pH

The pH of the litter was measured the same day of collection. To this end, the composite sample of litter was thoroughly mixed and 3 g was weighted, diluted in 60 ml of distilled water and vortexed. Samples were let to settle for 10 min and pH was measured from the supernatant with a GLP21 pH-meter (CRISON, Spain).

2.7.2. Litter moisture

The litter samples were processed the same day of collection. From the thoroughly mixed composite sample of litter, a 5 g sample was weighted, dried at 105 ºC for 24 h in a drying oven (J.P. Selecta, Spain), cooled down and weighted again, in order to measure the moisture (%). The litter moisture content (%) was calculated as follows: **[**(Wet litter weight-Dry Litter Weight)/Wet Litter Weight**]** x 100.

2.7.3. Relative humidity and temperature

At each farm visit, the relative humidity (RH) and maximum and minimum temperature inside and outside the broiler house were recorded from the control panel located in the anteroom of the broiler house.

2.8. Statistical analysis

The event of infection by *Campylobacter* spp. in the studied batches was assessed by means of a logistic regression model, including the farm as a random effect. Covariates included in all models were type of bed, type of drinker, type of ventilation, season at bird placement, maximum and minimum RH of the bed, maximum and minimum pH of the bed, maximum and minimum outdoor as well as indoor temperature, maximum and minimum outdoor and indoor RH. All results were obtained using GLIMMIX procedure of the SAS System V.9.3 (SAS Institute Inc.). Significance level was fixed at 5%.

3. Results

Overall, 63 flocks were monitored (12 or 13 flocks per farm) in the five studied farms and 61.90% (39/63) of flocks were *Campylobacter* positive before slaughter. None of the farms remained consistently negative throughout the study period (Table 2). Overall prevalence per farm (isolation of campylobacters from cloacal swabs) was 53.85%, 69.23%, 66.67%, 38.46%, and 83.33% in farms 1 to 5, respectively. Boot sock sampling allowed the early detection of *Campylobacter* in all five farms. The average age at which flocks became positive was 21.74 d (boot sock detection), with the earliest detection of *Campylobacter* being at 7 d of age from boot socks (four samples in three farms; farms 2, 3 and 4) and at 10 d from cloacal swabs (one flock in farm 4) (Table 2). In all farms at least one flock had positive birds at 14 d, with an overall of 9 flocks being positive at 10-16 d.

Both *C. jejuni* and *C. coli* were isolated in all farms, with up to three flocks colonized with both bacteria in all but one farm (Table 3). *C. jejuni* was, however, the most commonly isolated species in four out of five farms.

The day before and the same day of chick placement, boot sock sampling of the study house was performed, in order to confirm that no *Campylobacter* was present. All these samples in all five farms were negative throughout the study. Also, to verify that one-day old chicks were *Campylobacter* negative, lining paper soiled with faeces from crates in which chicks were transported were analysed, and all samples were also negative.

Only three boot sock samples from the path leading to the study house were positive (1.06%, 3/283); two in farm 1, with the broiler houses remaining negative throughout the rearing cycle, and one in farm 4, at the same time as the neighbour house, but the study house remained negative throughout the rearing cycle (Table 2). Five positive samples were detected in the anterooms (1.76%, 5/283) at 15d, 21d and 32d: one in farm 1 at 32d, at the end of the rearing cycle with the flock being negative until slaughter, one in farm 2 at 14d at the same time

as the birds from the study house, and three in farm 5 before or at the same time as in the broiler houses at 15d and 21d. Among the 5 farms, three of them were 2-house farms, and boot sock positive samples were detected in all neighbour houses at least in two flocks, in addition to the study house (5.19%, 11/212): two positives in farm 1, two positives in farm 4, and seven positives in farm 5. Positives in the neighbour houses were usually detected before or at the same time as in the study houses. Farm 5 was the one with an overall higher *Campylobacter* prevalence throughout the study in both the study house and the neighbour house, and the one with the higher number of positive samples in the anteroom of the study house.

None of the 283 water samples tested was positive to *Campylobacter*. Only three samples (0.86%, 3/349) of feed were positive; those were in farm 2 (two positives in flock 9, at the same time *Campylobacter* was first detected in boot socks and in cloacal swabs) and farm 4 (flock 8, before *Campylobacter* detection in boot socks or cloacal swabs).

In order to determine the speed of *Campylobacter* dissemination within a flock, once a cloacal swab was positive the farm was visited immediately for subsequent random spatial sampling of 30 birds. The flock was considered positive once all 30 cloacal swabs were positive. Frequently, at first sampling of 30 birds, all swabs were positive and the speed of colonization could not be determined. In those flocks were more than one visit to farms could be performed before all 30 cloacal swabs were positive, the minimum timing for a flock becoming colonized was just two days (farm 2). In all farms several flocks were colonized in only 3-4 days. The maximum time for a flock becoming colonized was 13 days (farm 3, flock 12).

Descriptive statistics is detailed in Table 4, with both qualitative data (infected flocks per farm, type of bed, type of drinker, type of ventilation and season at bird placement) and quantitative data (bed maximum and minimum relative humidity (RH) and pH, outside and inside maximum and minimum temperature and RH). Logistic regression model estimates are

shown in table 5, where infection with *Campylobacter* spp was more likely under higher minimal indoor temperature (β= 0.452; P=0.008) as well as higher minimal outdoor RH (β= 0.106; P=0.005). Furthermore, a significant effect of type of ventilation was found (P= 0.021), where infection was more likely in those farms with natural ventilation (natural air circulation system) than with either kind of forced ventilation, transversal and tunnel (OR=45.20, CIOR=3.18 to 643.20) or transversal (OR=8.20, CIOR=1.08 to 62.02). No influence of any other variable tested (type of drinker, type of bed, RH and pH of the bed, season at bird placement, maximum indoor and outdoor temperature and RH) was observed on *Campylobacter* colonization of flocks.

4. Discussion

This long-term longitudinal study with an intensive sampling allowed to analyse in detail the dynamics of *Campylobacter* in broiler farms in a country with a warm climate. It has been described that chickens remain free of *Campylobacter* the first two weeks of age, probably due to the presence of maternal antibodies and favourable environment (Evans and Sayers, 2000; Stern *et al*., 2001, Ridley *et al*., 2011; Perez-Arnedo et al., 2019). Accordingly, throughout the 2-years sampling the most common age at which we detected and isolated *Campylobacter* from birds (cloacal swab sampling) for the first time was 14 days. However, the time at which a bird first became colonized was significantly earlier (7-8 days) than the two weeks frequently reported in the literature (Ingresa-Capaccioni *et al*., 2016; Jacobs-Reitsma *et al*., 1995). This result may reflect the highly sensitive method (boot sock sampling and PCR detection) used in this study (Matt *et al.*, 2016). Our results are in accordance with other reports also using a PCR-based detection method, where *Campylobacter* could be detected in flocks younger than one week, suggesting an early colonization with low cfu numbers below the detection limit of culturing (Damjanova *et al*., 2011; Idris *et al*., 2006).

Campylobacter-positive boot socks sampled outside the broiler houses confirmed that *Campylobacter* is present in the surrounding environment of the farms, which can play a role in the ingress and dissemination of the pathogen into broiler farms (Guerin *et al*., 2007). However, the notably low prevalence found in these samples suggests that this is not the main source of *Campylobacter*. On the other hand, the simultaneous detection of *Campylobacter* in the study and neighbour rooms, or positive samples in the anteroom at the same time as the study house or the neighbour house, points out to a flaw or inadequate biosecurity at house level. This is in agreement with previous studies that report that *Campylobacter* is present in footwear, clothes, tools and other work equipment, as well as on workers hands or shoes, which represent an important source of contamination among broiler houses (Hald *et al.*, 2001). Also, since the anteroom and main doors are critical barriers, special attention to this area must be given when cleaning and disinfecting during the downtime (Ellis-Iversen *et al*., 2012).

Campylobacter can be found on the farm prior to a new flock arriving at the broiler house. Inadequate cleaning and disinfection and short downtime of the broiler house between flocks may be a major source of *Campylobacter* carryover (Agunos *et al*., 2014). However, in our study no *Campylobacter* was detected neither prior to chick placement, nor in one-day-old chicks, regardless of the previous flock being *Campylobacter*-positive or negative, which indicates adequate procedures of cleaning and disinfection between flocks.

Almost all positive boot socks in the study house led to a subsequent isolation of *Campylobacter* from cloacal swabs. Both *C. jejuni* and *C. coli* were isolated in all farms, being the former the most frequently detected. However, the *C. coli* prevalence was notably higher than that reported in broiler farms in northern Spain (García-Sánchez et al., 2020) and in most of other European countries (EFSA and ECDC, 2013). The very few negative cultures when the boot socks were positive suggest that *Campylobacter* could be present in a viable but nonculturable state (Battersby *et al*., 2016). Another possible explanation could be a side effect

of the therapeutic use of antibiotics in those flocks when it was needed, if for example infections by Gram-negative bacteria occur. Antibiotics are administered during the breeding period to control infections such as those caused by Avian Pathogenic *Escherichia coli* (APEC) (Refrégier *et al.,* 2001), which indirectly may alter *Campylobacter* colonization, reducing its bacterial load below the limit of detection by culture methods. On the other hand, although unlikely, a possible false positive PCR test result cannot be ruled out.

Caecal contents are commonly the sample of choice for *Campylobacter* detection and isolation, since it is where these bacteria accumulate the most in the gut (Lee and Newell, 2006; Allen *et al.,* 2007). However, in a previous study, we determined that caecal contents and cloacal swabs were equivalent in sensitivity for the early *Campylobacter* detection and isolation on farm (Urdaneta *et al*., 2015). Thus, in this study cloacal swabs were used, since it avoids bird sacrifice and saves time in sample collection and processing at the laboratory. Once a boot sock or a cloacal swab was *Campylobacter*-positive, the intensive sampling allowed us to determine more precisely the speed of transmission within a flock in natural conditions. Thus, it was determined that a whole flock can become colonized as fast as in 2-4 days. This is faster to what has been previously reported, which was less than one week (Ringoir *et al.,* 2007) or 4.4 to 7.2 days after colonization of the first broiler (van Gerwe *et al*., 2009). Overall, between 2 to 13 days was the time required for a flock becoming colonized. These results support previous studies which report that the time of colonization may vary between flocks, influenced by several factors that are still not clear (Cogan et al., 2007). Coprophagy is presumably a determinant factor in the fast dissemination of *Campylobacter* in the farms (Shreeve *et al.*, 2000; Newell and Fearnley, 2003). Other factors such as fluctuation of the RH and temperature, as well as the immunological condition of the birds, may also have an effect in the dynamics of *Campylobacter* colonization (Line, 2006; Cogan *et al.*, 2007).

Horizontal and vertical transmission has been pointed out as responsible of *Campylobacter* dissemination. Horizontal transmission has been clearly identified by different authors (Hald *et al.*, 2004; McDowell *et al*., 2008; Zweifel *et al.*, 2008; Ridley *et al.*, 2011). On the contrary vertical transmission between reproductive flocks is still not clear (Cox *et al*., 2012; Callicott *et al.*, 2006). The detection of *Campylobacter* DNA (by qPCR) in one-day-old chicks in few flocks (although negative by conventional culture) has been reported, thus suggesting the possibility of a vertical transmission (Marín *et al*., 2015). In agreement with previous studies, the lack of *Campylobacter* DNA detection by PCR in one-day-old chicks in our study confirms that vertical transmission is not relevant in the dissemination of this pathogen (Callicott *et al.*, 2006; Newell and Fearnly, 2003; Battersby *et al.*, 2016).

Several specific risk factors may have accounted for the overall higher *Campylobacter* prevalence in some of the studied farms. The types of ventilation system influence the likelihood of *Campylobacter* colonization of broiler flocks. Natural ventilation, which consists of windows at each side across the length of the broiler house with natural air circulation, where the opening of the windows is controlled manually (thus requesting an extra human intervention) and windows are kept open most of the time, may have been at higher risk than those with forced ventilation. Also, certain features of the surroundings of each farm could play a role in the transmission of *Campylobacter* in these facilities. For example, farm 1 was placed next to a feed factory, close to a dog kennel facility, and it also had domestic cats circulating freely nearby the broiler houses. Moreover, farm 2 was close to cattle and swine farms. These two farms showed an overall high *Campylobacter* prevalence. All of these have been reported as risk factors either for a higher presence of insects in the farm facilities or as persistent reservoirs of *Campylobacter*, thus contributing to maintain the burden of the pathogen needed to re-infect future flocks (Ellis-Iversen et al., 2012; Hald *et al*., 2004 and 2008; Humphrey *et al*., 2007; Refrégier *et al*., 2001; Ridley *et al*., 2011). The farm that showed an overall lower *Campylobacter* prevalence (farm 4) was in fact the most modern one, with facilities and

management that allowed a better biosecurity. On the contrary, the farm with the highest *Campylobacter* prevalence (farm 5) was the farm with a higher number of positive samples in the anteroom and the neighbour house; these results points to a poor biosecurity which has led to this high *Campylobacter* flock prevalence. Besides, the natural ventilation of this farm together with its location in a windy area, may have posed this farm at a higher risk, with an increased ingress of insects through the inlets (Hald *et al*., 2008). Logistic regression model showed that natural ventilation favoured the *Campylobacter* colonization of birds, compared to forced ventilation. Natural air systems favour the entry of *Campylobacter* by wind (Olsen *et al*., 2009) or by vectors (Hald *et al.,* 2004; Templeton *et al,* 2006). Higher minimal indoor temperature and higher minimal outdoor RH were also detected as risk factors, which are environmental conditions characteristic of warm climates such as those from Mediterranean countries. It has been reported that *Campylobacter* decreases when temperature is above 20ºC (Smith *et al*., 2016). However, high temperatures may cause stress in birds, which express higher levels of catecholamines (norepinephrine and epinephrine) in the blood, affecting the immune status of the animal by increasing susceptibility to infections (Line *et al.,* 2006; Cogan *et al*., 2007). On the other hand, RH may affect *Campylobacter* survival since these bacteria are sensitive to desiccation.

Water quality and its source have been reported as risk factors for the colonization of *Campylobacter* (Newell and Fearnley; 2003; Guerin *et al*., 2007; Sparks, 2009). Disinfection of water mainly by chlorine is important in order to inhibit all potential pathogens present in the water (Newell, 2002). In our study, all water samples analysed were negative to *Campylobacter*. Thus, the disinfection procedures used in the different farms were successful for the elimination of potential campylobacters in the water. With regards to the feed samples, only 0.86% were positive, thus confirming what has been reported that feed is not considered as a major source of contamination or introduction of *Campylobacter* (Wassenaar *et al.,* 2011). Unfortunately, it was not possible to confirm in those positive samples if the feed was contaminated before or after

the entry of birds in the broiler farm. Despite ample research, the epidemiology of *Campylobacter* spp. in commercial broiler production is not fully understood. The results of the present study contribute to gain insight into *Campylobacter* epidemiology, especially at farm level that may be relevant to other countries, especially those with similar climatic conditions.

In conclusion, several factors influence the presence of *Campylobacter* in broiler farms, but not all factors have the same relevance in the entry, colonization and dissemination of *Campylobacter*. In the present study, sampling methods allowed the earliest detection reported to date of a bird being naturally colonized by *Campylobacter*. Also, results show that a proper cleaning and disinfection during downtime are important in order to guarantee the complete removal of *Campylobacter* from the broiler house if the flock has been colonized. This is crucial to warrant starting a new rearing cycle free of *Campylobacter* because of the horizontal transmission of the pathogen. Besides, strict but achievable biosecurity measures both at farm and house level are needed in commercial broiler farms, in order to prevent flock colonization and transmission and thus reduce the current high flock prevalence. This is particularly important in poultry production in temperate regions with a less marked seasonality and therefore a wider period of higher risk of positive flocks. However, a combination of measures that may act synergistically, with enhanced biosecurity and hygiene as pillars of on-farm interventions are needed (Wales *et al*., 2019; Lu *et al*., 2021). These could include the combination of varied dietary approaches using feed additives and the acidification of drinking water. These potentially effective measures need, however, of large-scale field studies to provide robust evidence of their efficacy.

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Conflict of interest statement

The authors declare they have no conflicts of interest.

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Table legends

Table 1. Characteristics of the broiler farms included in the study.

Table 2. *Campylobacter* detection from birds and environmental samples by PCR and direct culture.

Table 3. *Campylobacter* species isolated in the five studied farms.

Table 4. Descriptive statistics of broiler house parameters assessed in the five studied farms.

Table 5. Parameters estimation of the logistic regression model with the event of infection by *Campylobacter* spp. as response variable.

Table 1.

Farm	No. houses	House age (yr)	Ventilation [a]	Size [b]	Capacity [c]	Bed type
1	2	>15	natural	1120 m^2	12,900 – 15,500	straw / wood shavings
2	1	10-15	forced	940 m^2	13,000 – 15,000	straw / wood shavings
3	1	6-10	forced	1428 m^2	20,200 – 25,596	wood shavings
4	2	2-5	forced	2900 m^2	40,000 – 46,200	wood shavings
5	2	>15	natural	2190 m^2	24,052 – 28,500	rice husks

a. Natural: natural air circulation system. Forced: transversal forced ventilation.

b. Size of each broiler house

c. Capacity of each broiler house.

Table 2.

	Farm 1			Farm 2		Farm 3		Farm 4			Farm 5		
Flock [f]	NH[a]	SH[a]	B[b]	SH[a]	B[b]	SH[a]	B[b]	NH[a]	SH[a]	B[b]	NH[a]	SH[a]	B[b]
F1	–	3/6 (34d)	34/37 [c]	1/6 (20d)	27/34	0/6 (35d)	–	1/1 (24d)	3/6 (28d)	28/31	1/1 (23d)	6/6 (23d)	23/27
F2	–	6/6 (26d)	26/30	5/6 (14d)[d]	14/20	5/6 (21d)	21/25	–	4/6 (14d)	14/21	–	5/6 (28d)	28/28
F3	–	5/6 (35d)	35/35	0/6 (29d)	–	4/6 (20d)	20/20	–	0/6 (26d)	–	–	2/6 (33d)	33/33
F4	1/1 (14d)	5/6 (14d)	14/14	0/6 (31d)	–	0/6 (29d)	–	–	0/6 (31d)	–	1/1 (22d)	6/6 (22d)[d]	22/28
F5	–	0/6 (32d)	–	6/6 (39d)	39/39	6/6 (14d)	14/17	–	1/1 (8d)	14/20	1/1 (21d)	1/6 (16d)	16/21
F6	1/1 (26d)	4/6 (26d)	30/30	5/6 (28d)	28/33	0/6 (28d)	–	1/1 (22d)	0/6 (28d)[e]	–	1/1 (14d)	3/6 (14d)[d]	–
F7	–	5/6 (28d)	28/31	1/6 (14d)	21/21	3/6 (29d)	29/37	–	0/6 (28d)	–	1/1 (20d)	6/6 (20d)	20/29
F8	–	1/6 (32d)[d]	–	0/6 (35d)	–	6/6 (31d)	31/31	–	4/6 (27d)	27/30	–	0/6 (27d)	–
F9	–	1/6 (27d)	–	2/6 (14d)	18/22	5/6 (14d)	14/17	–	0/6 (33d)	–	1/1 (23d)	6/6 (23d)	23/28
F10	–	0/6 (36d)[e]	–	1/1 (7d)	–	1/1 (7d)	14/14	–	0/6 (36d)	–	–	6/6 (21d)[d]	21/25
F11	–	0/6 (32d)[e]	–	5/6 (36d)	36/39	0/6 (28d)	–	–	0/6 (34d)	–	–	6/6 (20d)	20/31
F12	–	6/6 (19d)	19/22	6/6 (22d)	21/23	6/6 (20d)	20/33	–	1/1 (7d)	10/18	1/1(20d)	5/6 (20d)	20/29
F13	–	0/6 (27d)	–	6/6 (21d)	21/26			–	0/6 (27d)	–			

[a] Boot sock samples (PCR detection): NH, neighbour house; SH, study house; "-", *Campylobacter*-negative sample; for positive samples: number of positives/ total samples (age when a positive was detected).

[b] *Campylobacte*r detection by direct culture from cloacal swabs: B, birds from study house.

[c] Age (days old) 1st detection of a positive bird / age 30 birds positive from cloacal swabs.

[d] Flocks in which the anteroom was positive (boot socks): Farm 1: flock 8, 32d; Farm 2: flock 2, 14d; Farm 5: flock 4, 15d; flock 6 and 10, 21d.

[e] Flocks in which the path leading to the study house was positive (boot socks): Farm 1: flock 10, 21d and flock 11, 15d; Farm 4: flock 6, 22d.

[f] All water samples were negative. Feed samples positive: Farm 2, flock 9, 14d and 18d; Farm 4, flock 8, 21d.

Table 3.

Farm	*C. jejuni* [a]	*C. coli*	Mixed infections
1	28.57% (2/7)	57.14% (4/7)	14.28% (1/7)
2	55.55% (5/9)	11.11% (1/8)	33.33% (3/9)
3	75.00% (6/8)	25.00% (2/8)	0
4	80.00% (4/5)	0	20.00% (1/5)
5	80.00% (8/10)	0	20.00% (2/10)

a. Frequency (%) of flocks colonized by *C. jejuni*, *C. coli*, or *C. jejuni* and *C. coli* (mixed infections). In parentheses, number of colonized flocks/total infected flocks.

Table 4.

Parameters	Infected	Not infected	Total
Infected flocks per farm, n (%)			
Farm 1	7 (11.3)	6 (9.7)	13 (20.9)
Farm 2	8 (14.5)	4 (6.5)	12 (19.4)
Farm 3	8 (12.9)	4 (6.5)	12 (19.4)
Farm 4	5 (8)	8 (12.9)	13 (20.9)
Farm 5	10 (16.1)	2 (3.2)	12 (19.4)
Type of bed, n (%)			
wood shavings	17 (27.4)	9 (14.5)	26 (41.9)
rice husks	10 (16.1)	2 (3.2)	12 (19.4)
straw	11 (17.7)	10 (16.1)	21 (33.8)
Type of drinker, n (%)			
nipple with cup	31 (50)	18 (29)	49 (79)
nipple without cup	9 (14.5)	4 (6.5)	13 (21)
Type of ventilation, n (%)			
natural	19 (30.6)	6 (9.7)	25 (40.3)
forced transv.	16 (25.8)	8 (12.9)	24 (38.7)
forced transv.+ tunnel	5 (8)	8 (12.9)	13 (21.0)
Season at bird placement, n (%)			
winter	6 (9.7)	7 (11.3)	13 (21)
autum	12 (19.4)	5 (8)	17 (27.4)
spring	11 (17.7)	4 (6.5)	15 (24.2)
summer	11 (17.7)	6 (9.7)	17 (27.4)
Bed max RH [a]	42.6 ± 9.7	45.7 ± 11.3	43.7 ± 10.3
Bed min RH	11.5 ± 4.5	11.5 ± 4.0	11.5 ± 4.3
Bed max pH [a]	8.4 ± 0.9	8.6 ± 0.8	8.5 ± 0.9
Bed min pH	6.2 ± 0.4	6.1 ± 0.5	6.2 ± 0.4
Ext. max T [a]	22.9 ± 8.1	21.6 ± 9.6	22.5 ± 8.6
Ext. min T	12.6 ± 6.6	9.6 ± 9.8	11.6 ± 7.9
Ext. max RH [a]	70.6 ± 14.7	72.0 ± 14.4	71.1 ± 14.5
Ext. min RH	44.7 ± 11.9	39.6 ± 12.1	42.9 ± 12.1
Int. max T [a]	31.8 ± 1.6	31.6 ± 1.4	31.7 ± 1.6
Int. min T	24.2 ± 3.0	23.1 ± 3.7	23.8 ± 3.2
Int. max RH [a]	70.6 ± 9.3	71.3 ± 7.5	70.8 ± 8.6
Int. min RH	46.1 ± 10.6	46.0 ± 10.7	46.1 ± 10.5

a. Maximum and minimum relative humidity (RH) and pH of bed, and of temperature (T, ºC) and RH (%) outside (ext) and inside (int) the broiler houses.

Table 5.

Parameter [a]	Estimate	Standard error	Significance (P-value)
Intercept	-16.792	5.538	0.094
Type of ventilation			
natural	3.811	1.325	0.006
forced transv.	1.708	1.020	0.100
forced transv.+ tunnel	0		
Int. min T	0.452	0.165	0.008
Ext. min RH	0.106	0.036	0.005

a. Natural: natural air circulation system. Forced: transversal forced ventilation (with or without tunnel). Int. min T: minimal temperature inside the broiler houses. Ext. min RH: minimal relative humidity outside the broiler house.