Campylobacter prevalence and risk factors in poultry slaughterhouses in Spain following the EU criteria

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10 **ABSTRACT**

11 Background: *Campylobacter* is the main pathogen involved in zoonotic gastrointestinal
12 diseases. Last year, the European regulation 2017/1495 on *Campylobacter* in broiler
13 carcasses came into force. In this context, the aim of this study was to assess the potential
14 risk factors associated with exceeding the 1,000 CFU/g criterion set by the European
15 Commission in several slaughterhouses in Spain.

16 Methods: Information relating to 12 factors were collected using questionnaires. Samples
17 were collected from 12 Spanish abattoirs during June, July and August 2017 (n=1,725)
18 and were analysed following ISO/TS 10272-2:2006 method.

19 Results: The proportion of *Campylobacter*-positive samples was 23.7% (n=409).
20 Analysis of the flock age (41-50 days) revealed a significantly increased OR in
21 *Campylobacter* enumeration (OR=7.41). Moreover, scalding temperature (51.9.54 ºC)
22 was positively associated with an increase in OR (OR=2.75). Time in transit to slaughter
23 (1-1.5h), showed a significant OR decrease (OR=0.25). However, when processed for
24 more than 2 hours, presented an increase in OR (OR=4.44). Regarding carcass weight,
25 the range from 3.21-3.58 presented a decrease in OR (OR=0.01).

26 Conclusion: The outcomes of this study suggest that although most chickens are
27 contaminated by the bacterium, the prevalence that exceeds the limit of 1,000 CFU/ is not
28 so high as we thought.

29 **Key words**: *Campylobacter*, poultry, slaughterhouse, quantitative, risk factor

INTRODUCTION

Campylobacter is the main bacterial cause of human gastroenteritis in most industrialised countries and has been since 2005 [1-3]. In 2017, campylobacteriosis was the most commonly reported zoonosis, representing almost 70% of all cases [4]. The European Surveillance System described 246,158 confirmed cases of human campylobacteriosis in EU. However, their severity in terms of reported case fatality was low (0.04%) [4].

Consuming undercooked poultry meat and cross-contamination due to a lack of hygiene conditions are the main sources of campylobacteriosis outbreaks. Several studies have shown *Campylobacter* prevalence in poultry carcasses. Lawes et al. (2012) observed that 70% of poultry carcasses in the UK were contaminated with the bacterium [5]. Moreover, a qualitative cross-contamination study showed that *Campylobacter* was easily transferred from raw chicken products to cutting boards, plates and particularly to hands [6].

To lower the risk of human infection, in the drive to reduce the number of flocks colonised with this bacterium most studies have focused on rearing at poultry farm level [7-9]. Nevertheless, the epidemiology of *Campylobacter* in poultry production is still not fully understood, making it difficult to control [10].

In this context, biosecurity plans, immunity and nutritional strategies have been studied. However, none of these measures has managed to reduce *Campylobacter* prevalence to acceptable levels [11].

Even though *Campylobacter* is the main pathogen involved in zoonotic gastrointestinal diseases, there is no national or European control programme at farm level, in contrast to *Salmonella*, probably due to the incomplete knowledge of *Campylobacter* epidemiology. In this regard, legislators have been working to limit *Campylobacter* presence in broiler carcasses. To this end, earlier this year, European regulation 2017/1495 on

55 *Campylobacter* in broiler carcasses came into force. Under this new European regulation,
56 neck skin from broiler carcasses at the slaughterhouse must be analysed for
57 *Campylobacter* after chilling with the microbiological criterion of maximum 1,000
58 CFU/g (colony form units/per gram) [12].
59 It has been suggested that the pre-slaughter handling and transport of broiler chickens are
60 important stress factors for bacteria shedding [13]. Poultry carcasses could be
61 contaminated with the bacterium due to splashing of intestinal contents during the
62 slaughtering process. Mainly the stages of scalding, de-feathering and evisceration could
63 increase the contamination of carcasses [14,15], representing critical points where the
64 *Campylobacter* counts can be reduced.
65 In this context, the aim of this study was to assess the potential risk factors associated
66 with exceeding the 1,000 CFU/g criterion set by the European Commission in several
67 slaughterhouses in Spain.

MATERIAL AND METHODS

Study sample

This study was carried out on 12 Spanish commercial broiler poultry processing plants which slaughter close to 20% of the chickens reared in Spain. The processing line was operating under standard commercial conditions. Over three months (from June to August 2017), three visits were made to each processing plant, with a total of 36 visits. At each visit, the first and the last flock processed were sampled in order to assess cross-contamination. Characteristics of the slaughterhouses are listed in Table 1.

Table 1. Characteristics of each slaughterhouse.

Variable	Slaughterhouse											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Number of animals slaughtered (total)	10,001-30,000	1,000-30,000	1,000-10,000	1,000-10,000	20,001-30,000	1.000-10.000	30,000-40,000	80,001-100,000	20,001-30,000	10,001-30,000	80,001-100,000	1000-10,000
Flock size animals	4,001-5,000	4,001-5,000	500-2,500	1,001-2,000	2501-6500	500-6,500	2501-6500	4,501-8550	4501-8550	500-8500	2501-6500	500-2,500
Slaughter age	41-50	41-50	71-80	71-100	41-50	41-60	30-50	30-50	30-60	30-50	30-60	81-90
Scalding temperature (ºC)	48-51.8	48-51.8	48-51.8	48-51.8	48-51.8	48-54	48-54	51.9-54	48-54	48-54	51.8-54	48-51.8
Amount of chlorine (ppm)	0-0.5	0-0.5	0.5	0.5	0.51-0.95	0.51-0.95	0.51-0.95	0.51-0.95	0-0.95	0-0.5	0-0.5	0-0.5
Conservation temperature (ºC)	≤0	≤0	≤0	≤0	>0	>0	>0	≤0	>0	>0	>0	≤0
Chilling time (min)	60-100	151-200	>300	251->300	60-100	60-300	101-150	101-150	101-150	60-100	201-250	60-100
Time in transit to slaughter (h)	1.6-2	1-1.5	1.6->2	1-1.5	<1	1-2	1-1.5	<1-1.5	<1-2	<1->2	<1	1-1.5
Carcass weight (kg)	1-3	2.51-3	>3	>3	2.01-3	2.01-3	1-3	1-3	2.01->3	1-2.5	2.51->3	>3

Sampling during slaughtering

From each studied flock, samples were collected at five selected stages of the processing line (after scalding, after de-feathering, after eviscerating, after washing and after chilling) to determine the degree of *Campylobacter* contamination in the carcasses throughout the processing line (figure 1). In each stage, five carcasses were collected (25 carcasses per flock), which means 50 carcasses per sampling session, 25 from the first flock and 25 from the last flock. All samples were transported under cold storage conditions and analysed within 24h.

Carcass sampling

Sampling of carcasses was carried out by collecting neck skin with aseptic conditions and placing them in an individual sterile bag (Seward, Worthing, UK). Neck skin samples were obtained from each carcass by removing a strip of neck skin (25g) with a sterile scalpel and tweezers. Samples were transported to the laboratory on the same day of sampling. Each individual skin sample was placed in a sterile bag and diluted at 1:10 vol/vol Buffered Peptone Water (BPW, Scharlau®, Barcelona, Spain). The mix was homogenised by stomaching at 230 rpm for 120 seconds (Stomacher®400 circulator, Seward Ltd., Worthing, UK). Then, 1 ml of the homogenate was used for *Campylobacter* enumeration.

Campylobacter enumeration

Neck skin homogenates were analysed according to the ISO/TS 10272-2:2017, the horizontal method for the enumeration of *Campylobacter* in food and feed stuffs [16]. For detection purposes, serial dilutions were prepared in BPW and a 0.1 mL drop from each inoculum was plated onto mCCDA (Modified Charcoal Cefoperazone Deoxycholate

104 Agar, Oxoid, Dardilly, France). The samples were then incubated at 41.5 ± 1°C in a
105 microaerobic atmosphere (84% N_2, 10% CO_2, 6% O_2) for 44 ± 4 hours. Five
106 *Campylobacter*-like colonies were plated in Columbia blood agar (AES Laboratories®,
107 Bruz Cedex, France) for further characterisation. Colony morphology and motility were
108 evaluated under dark field microscopy. Confirmation of the suspicious colonies was
109 performed by oxidase and catalase tests and plating at different temperatures and
110 atmospheres (41.5 ºC under microaerophilic conditions and 25 ºC under aerobic
111 conditions) onto Columbia blood agar (AES Laboratories®, Bruz Cedex, France).
112
113 ***Study critical points***
114 Every sampling was accompanied by a questionnaire that included the information on the
115 slaughterhouse, month of sacrifice, position of the batch in the daily slaughter schedule,
116 processing stage at sampling, total number of animals slaughtered, flock size, age of
117 animals at slaughter, scalding temperature, amount of chlorine, chilling time and
118 conservation temperature, time in transit to slaughter and carcass weight. The variable
119 levels studied are represented in Table 2.

120 **Table 2.** Number of variables assessed during the sampling period.

Variable	Level	Reference
Slaughterhouse	1 2 3 4 5 6 7 8 9 10 11 12	Lawes et al. (2012)
Month	June July August	Lawes et al. (2012)
Batch position	1st 2nd	Hue et al. (2010)
Processing stages	After scalding After de-feathering After eviscerating After washing After chilling	Guerin et al. (2010)
Number of animals slaughtered (total)	1,000-10,000 10,001-20,000 20,001-30,000 30,001-40,000 40,001-80,000 80,001-100,000	
Flock Size (animals)	500-2,500 2,501-4,500 4,501-6,500 6,501-8,550	Näther et al. (2009)
Slaughter age (days)	30-40 41-50 51-100	Näther et al. (2009)
Scalding temperature (ºC)	48-51.8 51.9-54	Hue et al. (2010)
Amount of chlorine (ppm)	0-0.5 0.51-0.95	Vinueza-Burgos et al. (2017)
Conservation temperature (ºC)	≤0 >0	Hue et al. (2010)
Chilling time (min)	60-100 101-150 151-360	Hue et al. (2010)
Time in transit to slaughter (h)	<1 1-1.5 1.6-2	
Carcass weight (kg)	1-2,6 2.61-3.2 3.21-3.58	

121

122 ***Statistical analysis***

123 Differences in *Campylobacter* contamination levels at slaughterhouses and processing
124 stages (after scalding, after de-feathering, after eviscerating, after washing and after
125 chilling) were assessed by the Chi-square test (at 95% CI and $p<0.05$).

126 The binary outcome was created to investigate slaughterhouse level factors associated
127 with the presence of *Campylobacter* (1 if the sample exceeded the criterion of 1,000
128 CFU/g and 0 if *Campylobacter* enumeration was below 1,000 CFU/g). Multivariable mix-
129 effect logistic regression was conducted to identify factors associated with
130 slaughterhouses, reporting the odds ratio (OR) and 95% confidence intervals (CI).
131 Slaughterhouse was included as a random effect in all analyses. A total of 12 exposure
132 variables (listed in Table 1) were evaluated as fixed effects in univariable analyses.
133 Variables with a univariable p-value of <0.10 were considered for inclusion in a
134 multivariable model, which was built using a forward stepwise approach. A separate
135 analysis was also conducted to investigate risk factors associated with *Campylobacter* on
136 carcasses after chilling. A total of 11 exposure variables (listed in Table 1, with the
137 exception of processing stages) were evaluated as fixed effects in univariable analyses.
138 As previously, variables with a univariable p-value of <0.10 were considered for inclusion
139 in a multivariable model, which was built using a forward stepwise approach. Models
140 were run using Stata 13 (College Station, Texas, USA) and the 'xtmelogit' command.

141

142 **RESULTS**

143 ***Samples that exceeded the 1,000 CFU/g criterion during slaughtering***

144 During the study, a total of 1,725 samples were collected from the processing line of 12
145 different slaughterhouses for *Campylobacter* enumeration. Seventy-five samples from 1
146 slaughter were discarded due to sampling conditions. Of this amount (n=1,725), the

147 23.7% (n = 409) exceeded the 1,000 CFU/g criterion. Statistical differences were shown
148 in *Campylobacter* counts according to different processing stages (after scalding, after
149 de-feathering, after eviscerating, after washing and after chilling) and the different
150 slaughterhouse studied (Figure 2 and 3). Concerning sampling at each processing stage,
151 the higher counts were shown after the evisceration stage, where 29% of samples
152 exceeded the limit of 1,000 CFU/g (n = 100/345) and the lowest one after chilling stage
153 (19.1%, n = 66/345) (Figure 2).
154
155 In terms of critical points, significant differences were found in the processing phases, as
156 the stages after washing and after chilling showed a decrease in the OR (OR = 0.63, *P* =
157 0.031 and OR = 0.58, *P* = 0.013, respectively). The total animals slaughtered ranging
158 from 10,001-20,000 showed an increase in the OR (OR = 2.5, *P* = 0.010). However, those
159 ranging from 30,001-40,000 presented a decrease in the OR (OR = 0.08, *P* = 0.000).
160 Moreover, significant differences were found in the flock size, with the 6,501-8,500 range
161 presenting the decreased OR (OR = 0.04; *P* = 0.000). The age of conventional birds (41-
162 50 days) was positively associated with a higher *Campylobacter* count (OR = 2.37, *P* =
163 0.000). With reference to scalding temperatures, the 51.9-54ºC range showed an OR
164 increase (OR = 3.99, *P* = 0.000). Moreover, *Campylobacter* was isolated more in samples
165 chilled at temperatures above 0ºC (OR = 4.82, *P* = 0.000). In addition, the chilling time
166 range between 101-150 presented an increased OR (OR = 2.00, *P* = 0.000). Transit time
167 to slaughter of more than 1 hour presented a significant increase in the OR (1-1.5 h; OR
168 = 3.20, *P* = 0.000), (1.6-2 h; OR = 13.34, *P* = 0.000) and (>2 h; OR = 5.91, *P* = 0.000).
169 With respect to the weight of carcasses (3.21-3.58 kg), the range presented a significant
170 decrease in the OR (OR = 0.32, *P* = 0.003) (Table 3).
171

Table 3. Final multivariate mixed-effect logistic regression of risk factors associated with exceeding the 1,000 CFU/g criterion during all slaughterhouse stages. Slaughterhouse was included as random effect.

Variable	Level	Odds ratio	*P-value*	95% CI
Processing stages	After scalding	Ref.		
	After de-feathering	0.88	0.547	0.59-1.31
	After eviscerating	1.21	0.328	0.82-1.77
	After washing	0.63	0.031	0.42-0.95
	After chilling	0.58	0.013	0.39-0.89
Number of animals slaughtered (total)	1,000-10,000	Ref.		
	10,001-20,000	2.54	0.010	1.25-5.19
	20,001-30,000	1.66	0.074	0.95-2.89
	30,001-40,000	0.08	0.000	0.03-0.20
	40,001-80,000	0.51	0.226	0.17-1.51
	80,001-100,000	0.71	0.457	0.28-1.74
Flock Size (animals)	500-2,500	Ref.		
	2,501-4,500	0.19	0.000	0.10-0.35
	4,501-6,500	0.24	0.000	0.13-0.44
	6,501-8,550	0.04	0.000	0.01-0.11
Slaughter age (days)	30-40	Ref.		
	41-50	2.37	0.000	1.46-3.85
	51-100	1.15	0.747	0.47-2.84
Scalding temperature (ºC)	48-51.8	Ref.		
	51.9-54	3.99	0.000	2.59-6.16
Conservation temperature (ºC)	≤0	Ref.		
	>0	4.82	0.000	2.87-8.09
Chilling time (min)	60-100	Ref.		
	101-150	2.00	0.000	1.47-2.71
	151-360	1.00	0.999	0.67-1.49
Time in transit to slaughter (h)	<1	Ref.		
	1-1.5	3.20	0.000	1.81-5.63
	1.6-2	13.34	0.000	6.04-29.44
	>2	5.91	0.000	2.96-11.81
Carcass weight (kg)	1-2,6	Ref.		
	2.61-3.2	0.50	0.000	0.35-0.71
	3.21-3.58	0.32	0.003	0.15-0.68

Ref.: Reference. CI: Confident Interval.

Samples that exceeded the 1,000 CFU/g criterion after chilling

Eleven variables were tested and four showed an association with the *Campylobacter* count during the univariable analysis after chilling. Findings from the mixed-effects between different critical points and the samples that exceeded the criterion of 1,000 CFU/g of *Campylobacter* after the chilling stage are summarised in Table 4. Analysis of the flock age revealed significantly increased OR in *Campylobacter* enumeration (OR = 7.41, *P* =

184 0.001). Similarly, scalding temperature (51.9.54 ºC) was positively associated with an
185 increase in the OR (OR = 2.75, *P* = 0.006). Examining the time in transit at slaughter there
186 was a significant OR decrease in samples processed for more than 1-1.5 hours (OR = 0.042,
187 *P* = 0.000). However, those processed for more than 2 hours presented an increase in the
188 OR (OR = 4.44, *P* = 0.050). Regarding carcass weight, the range from 3.21-3.58 presented
189 a decrease in the OR (OR = 0.01, *P* = 0.011).

190
191 **Table 4.** Final multivariate mixed-effect logistic regression of risk factors associated with
192 exceeding the criterion of 1,000 CFU/g after the chilling stage. Slaughterhouse was
193 included as random effect.
194

Variable	Level	Odds ratio	*P-value*	95% CI
Slaughter age	30-40	Ref.		
	41-50	7.41	0.001	2.19-25.02
	51-100	3.53	0.205	0.50-25.03
Scalding temperature (ºC)	48-51.8	Ref.		
	51.9-54	2.75	0.006	1.68-22.98
Time in transit to slaughter (hours)	<1	Ref.		
	1-1.5	0.25	0.042	0.06-0.95
	1.6-2	0.68	0.687	0.10-4.34
	>2	4.44	0.050	0.99-19.75
Carcass weight (kg)	1-2,6	Ref.		
	2.61-3.2	0.22	0.003	0.08-0.61
	3.21-3.58	0.01	0.011	0.00-0.03

195
196 Ref.: Reference. CI: Confident Interval.

197

198 **DISCUSSION**

199 Campylobacteriosis is the main zoonotic gastrointestinal disease in the EU and Spain
200 comes fourth in the list of countries reporting the majority of cases (18,860), after
201 Germany, the United Kingdom and the Czech Republic [4]. *Campylobacter* colonises the
202 poultry gut and the caecal contents of chicken and can contain extremely high
203 *Campylobacter* counts (10^9 CFU/g) [17]. Several authors have revealed that the
204 evisceration process leads to a considerable increase in cross-contamination of

205 *Campylobacter*, especially as perforation of the intestines often cannot be avoided [18-
206 20].
207 In this study, mCCDA medium was employed for *Campylobacter* counting, the medium
208 followed by the standard method [16]. However, it is a selective medium with low
209 discrimination of real *Campylobacter,* and for this reason the discrimination requires
210 trained and experienced personnel and time for confirmation tests (microscopic
211 observation, biochemical testing) [21]. Nevertheless, comparative studies with other
212 selective media such as CFA (Campy Food Agar), did not reveal statistically significant
213 differences between *Campylobacter* counts [21-22]
214 Our results showed an increased number of samples that exceeded the criterion of 1,000
215 CFU/g after evisceration stage, and a 34.5% decrease after the chilling stage. These
216 results are consistent with others published by Figueroa et al. (2009) and Guerin et al.
217 (2010), who observed a decrease of 26.6% and 17.5 %, respectively [21-22].
218 In contrast, no statistical differences were found for the batch position (first and last)
219 within the same slaughter, but statistical differences were observed between the different
220 slaughterhouses. This suggests that *Campylobacter* presence in the carcasses might be
221 more associated with the origin of the batch than its position in the slaughter schedule.
222 Although the entry of positive animals could result in cross-contamination of the
223 slaughterhouse, it has been reported that only the two subsequent batches are
224 contaminated in a positive lot [23]. However, the microbiological status of the previous
225 batch was not considered in the present study.
226
227 After the chilling stage, the following risk factors identified were the age of the animals,
228 scalding temperature, time in transit to the slaughterhouse and the carcass weight. A
229 higher percentage of samples exceeded the criterion of 1,000 CFU/g in flocks sacrificed

between 41-50 days of age. Lawes et al. (2012) showed an increase in *Campylobacter* prevalence in animals slaughtered at 46 days of age [5]. Nevertheless, Hue et al. (2010) showed a high prevalence of *Campylobacter* in older ages (free-range or slow growth) [24]. Studies suggest that *Campylobacter* broiler colonisation starts at the age of 21 days (2-3 wks.) and there is a significant increase during the fattening period (from 40 days onwards) [8].

Concerning the scalding temperature, our results presented an increase in the OR when samples were scalded at 51.9-54ºC. Procedures for *Campylobacter* control together with freezing have been shown to reduce the bacteria, although due to the pathogen's ability to survive in water, in aerosols and on equipment, cross-contamination can occur between batches, and carcasses free from *Campylobacter* can become contaminated after an infected batch of birds [23,26]. Regarding time in transit to slaughter, from 1-1.5 hours was associated with a decrease in *Campylobacter*. However, more than 2 hours tended to be associated with an increase in the *Campylobacter* presence. This could be due to the longer time the animals spent in the slaughterhouse, which may be insufficiently technically equipped and have less machinery (fewer plucking fingers, fewer showers and fewer evisceration devices). Therefore, more carcasses pass through the same machines, and more cross-contamination could occur between batches.

Regarding carcass weight, although higher levels of *Campylobacter* are reported when the animals are larger [27], in this case we found a protective factor when the animals weighed between 3.21-3.58 kg. However, other authors did not find differences between the body weight and subsequent proliferation of the bacteria [28].

Campylobacter is a confirmed pathogen from gastrointestinal content in poultry. Measures taken so far have not managed to control the bacteria at field level, as once the chickens are colonised they remain so until slaughter [31]. Thus, its control has been regulated at the slaughterhouse. Previous studies reported that reducing *Campylobacter* prevalence by two logs at field level could lead to a 30-fold reduction in human campylobacteriosis [31,32]. After that, by following control strategies such as biosecurity measures at field level together with optimisation of technical and hygienic aspects of the slaughterhouse, *Campylobacter* counts in chicken could be reduced.

Although some studies have reported a prevalence of 70% and 86% in broiler carcasses, the results of this study suggest that the prevalence that exceeds the limit of 1,000 CFU/g (limit of infection in humans) is low [5]. Nine of the twelve slaughterhouses analysed in the study met the criteria set out in the legislation (1,000 CFU/g; c=20; n=50). Direct comparison of these results to others reported is delicate and should be performed with caution due to the heterogeneity of the experimental design. To our best knowledge, this is the first study to examine the risk factor of *Campylobacter* at the slaughterhouse, taking the EU legislation into account.

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