MICROBIOLOGY AND FOOD SAFETY

Evaluation of effect of chilling steps during slaughtering on the *Campylobacter* sp. counts on broiler carcasses

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ABSTRACT *Campylobacter* spp. play an increasing role as foodborne pathogens, with poultry representing the main vehicle of infection, and control measures at the slaughterhouse have been implemented in the last years. In this study, 2 trials were performed, evaluating the effect of the chilling phases currently applied in an industrial slaughterhouse on the *Campylobacter* sp. contamination of broiler carcasses. In the first trial, neck skin samples were taken from 13 flocks before and after the on-chain air chilling and submitted to analysis of *Campylobacter* sp. count; in the second trial, 63 carcasses or cuts stored in the chilling room for variable times, with or without skin, were submitted to analysis of *Campylobacter* sp. count. A selection of 75 isolates was identified by PCR. All carcass skin samples taken from the first trial showed *Campylobacter* sp. counts higher than 0.7 log cfu/g. A wide variability in the counts (about 3 logs) was detected, showing a high correlation between the counts obtained before and after chilling. A slight decrease ($P = 0.011$) was observed after chilling (mean difference of about 0.3 log cfu/g), also if variability was observed among the flocks; the number of samples with high *Campylobacter* sp. counts (≥3 log cfu/g) was reduced ($P = 0.010$). In the second trial, low counts were generally detected (almost all lower than 3 log cfu/g). An evident decreasing trend was observed during storage, but the survival rate of *Campylobacter* on the cuts with skin was higher. All the isolates were identified as *Campylobacter jejuni* (72%) or *Campylobacter coli*. The data obtained were compared with the threshold limit set by EC Regulation 2073/2005, evidencing the impact of the sampling point on the counts. Our results highlighted the importance of applying a hurdle strategy including on-chain chilling and strict respect of the cold chain, allowing the food business operator to fulfill the process hygiene criteria and avoiding the delivery of highly contaminated meats.

Key words: broiler carcass, *Campylobacter* sp., on-chain chilling, process hygiene criteria

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INTRODUCTION

As widely described, campylobacteriosis represents the most widespread zoonosis in Europe, causing about 250,000 cases per year (EFSA, 2019); poultry and derived meat are considered the main vehicle of the infection, and their manipulation, preparation, and consumption are directly linked to the onset of 20–30% of human campylobacteriosis cases (EFSA, 2010b).

The efforts applied during the last decades to reduce the prevalence of *Salmonella* spp. in poultry and derived products, which led to a marked reduction of its prevalence in poultry population and of the rate of human cases, did not result in a parallel reduction in the distribution of *Campylobacter* or of human campylobacteriosis cases. This is argued to be due to a higher distribution of this microorganism in the animal population and to a higher number of possible vehicles of distribution at the farm level (Keener et al., 2004). The data from European Food Safety Authority (EFSA) indicate a prevalence of about 50% on slaughtered broiler carcasses, but with a wide variability (EFSA, 2010a).

In the final part of the production chain, it is very difficult to prevent the contamination of meat as the frequent presence of high counts in the gut and on the feathers leads to an unavoidable cross contamination among different animals or flocks during scalding, defeathering, and the further slaughtering and sectioning phases. Several interventions have been suggested to reduce the distribution of contamination, including interventions at the farm, during transport and slaughter, and at the

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consumer level (EFSA, 2011). Owing to the wide distribution of the pathogen, these interventions focus mainly on decreasing *Campylobacter* counts rather than elimination. As *Campylobacter* is not able to grow on the surface of carcasses or sectioned meats, the initial contamination is of particular interest to assure the absence of highly contaminated meats entering the consumer's kitchen, which can be a source of contamination of surfaces and equipment and proper food handling and subsequently of ready-to-eat foodstuffs. The importance of high *Campylobacter* counts on poultry meat in determining the risk for the consumers has been also highlighted by quantitative microbial risk assessment (Nauta and Havelaar, 2008).

It is well known that thermotolerant *Campylobacter* species are sensitive to the effect of harsh environments characterized by low temperatures, low humidity, and oxidizing atmosphere (Keener et al., 2004). Some studies showed a high survival rate at room temperature (Peyrat et al., 2008; Vandeplas et al., 2008), but a decreasing effect for chilling or freezing has been described, with a very marked effect of the latter, resulting in complete inactivation of these pathogens or in a significant reduction of counts (Lee et al., 1998; Sampers et al., 2010; Chapman et al., 2016).

The decontaminating effect of low temperatures is influenced by humidity (air chilling is more efficient than immersion chilling) (Sanchez et al., 2002), the initial *Campylobacter* contamination (Pearson et al., 1996), and the characteristics of the surface (a higher survival rate is reported on poultry skin than on the meat surface) (Davis and Conner, 2007).

The EC Regulation No. 2073/2005, as amended by the EU Regulation No. 1495/2017, requires the food business operator (**FBO**) to evaluate *Campylobacter* sp. counts on the neck skin of broiler carcasses as a process hygiene criterion from January 2018, with a limit of 1,000 cfu/g, and a tolerance of 15 of 50 carcasses (EU Regulation No. 2073/2005). Moreover, EU Regulation No. 627/2019 (EU [European Union], 2019) has recently implemented the official control procedures as *Campylobacter* is recognized as one of the main human health hazards to be covered by inspection of poultry meat. In light of the aforementioned information, it is particularly important for the FBO to apply proper procedures to limit the contamination of carcasses, as the FBO is responsible for hygiene condition of meat, independently from the source of live animal contamination.

The aim of the present study was the assessment of the effect of the chilling phases applied in an industrial broiler slaughterhouse to the *Campylobacter* sp. contamination level, evaluating the current situation in the light of the evolving process hygiene criterion.

MATERIALS AND METHODS

The Production Plant

The trials were performed in an industrial broiler chicken slaughterhouse, with a slaughtering rate of 35,000–40,000 animals per day. The slaughtering process includes the following phases: hanging, electrical stunning, sticking and bleeding, scalding (water bath, temperature of 49°C–50°C), defeathering, evisceration, washing (with potable water), and sectioning. Two chilling phases are applied during the process: the first is applied on hung carcasses, after the evisceration and washing phases and before sectioning; the carcasses are exposed to an air flux (with a variable speed of 0.5 to 2 m/s depending on the pint of the chain) for 35 min; air temperature is set at 2 ± 0.5°C, resulting in a decrease in air temperature from 13°C (entry of the carcasses) to 4°C (end of the process).

The second phase is applied to the carcasses or cuts before delivery to customers; the carcasses and or the cuts are kept in a static chilling room (air temperature of 2°C–3°C) for a variable time (1–7 d).

First trial: Evaluation of On-Chain Chilling of Whole Carcasses Before Sectioning

To evaluate the effect of the on-chain chilling phase on the *Campylobacter* sp. counts on the surface of carcasses, sampling was performed on 13 broiler pooled samples, with each pool belonging to a different slaughtering batch (corresponding to a specific flock) coming from different farms, during 3 replications.

The presence of *Campylobacter* sp.–positive broiler flocks was assessed in pooled cecal content samples, applying both a detection and count method. For detection of *Campylobacter* spp., the ceca were taken from 5 carcasses per flock just after carcass evisceration; the surface was disinfected and aseptically opened. Then, the cecal content (1–2 g for each cecum) was pooled into a sterile stomacher bag and diluted to a ratio of 1:10 with Bolton broth (Biogenetics, Ponte San Nicolò, Italy), and analyzed following the ISO 10272-1 method (ISO, 2006a): in brief, the broth was incubated for 48 h in microaerobic atmosphere (4 h at 37°C, followed by 44 h at 41.5°C) and inoculated using a sterile loop onto modified Charcoal Cefoperazone Deoxycholate Agar and Karmali agar plates (Scharlab, Barcelona, Spain), which was incubated at 41.5°C for 48 h in microaerobiosis.

The same sampling technique was applied to the counts; except, the pooled samples were submitted to 1:10 dilution with buffered peptone water (BPW; Scharlab). The samples were homogenized for 60 s in a Stomacher 400 (Seward Medical, London, UK); then, serial dilutions were prepared in BPW and spread onto modified Charcoal Cefoperazone Deoxycholate Agar plates, following the ISO 10272-2 method (ISO, 2006b). The limit of quantification was equal to 1 log cfu/g. For the confirmation of *Campylobacter* identification, the ISO 10272-1 protocol was applied.

For the evaluation of carcass contamination, 3 pooled samples (5 carcasses for each sample, not the same used for cecal content sampling) were taken from each flock both before and after chilling. Neck or breast skin

samples were taken using a sterile knife, as per the method described by EC Regulation 2073/2005 (as amended by the EU Regulation No. 1495/2017) (EU, 2005; EU, 2017). The samples were then put into a sterile bag and kept refrigerated (4°C) until the analysis, which was performed the same day, and diluted 5-fold with BPW, and the ISO 10272-2 method was applied; the limit of quantification was equal to 0.7 log cfu/g.

To monitor distribution of temperature of broiler carcasses during the chilling process, 2 temperature probes equipped with data loggers (Escort iLog, Aesch bei Birmensdorf, CH) were placed on selected carcasses (total carcasses: 2 per flock): for each carcass, a probe was applied to the skin surface (where *Campylobacter* is usually present in this phase), and the other was applied just beneath it (where the pathogen could be carried during the last slaughtering operations and sectioning). Different carcasses were sampled for microbiological analyses and temperature determination.

Aiming to monitor the distribution of the main *Campylobacter* species, the isolates from a selection of samples (a total of 28 isolates) were submitted for determination of the main *Campylobacter* species (presence of *Campylobacter jejuni* or *Campylobacter coli*), following the PCR method described by Denis et al. (1999, 2001).

Second Trial: Evaluation of Chilling of Carcasses or Cuts After Slaughtering

A total of 63 broiler whole carcass or cut samples were withdrawn from the chilling room of the slaughterhouse after different storage times (25 samples after maximum of 1 d from the slaughterhouse, 23 samples after 2 to 4 d, and 15 samples after more than 4 d). Samples with (n = 36: whole carcasses, legs, or thighs) and without skin (n = 27: whole breasts) were taken to evaluate the effect of refrigeration on different surfaces.

The samples were put in sterile bags, kept refrigerated (4°C), and transported to the laboratory, where they were analyzed within the same day. Sampling from skin-on cuts or whole carcasses was performed by taking a total of 25 g of skin, as performed for the hung carcasses (first trial), whereas muscle surface (maximum: 5-mm thick) was taken from samples without skin. The samples were submitted to the analyses, applying the *Campylobacter* sp. count method (ISO 10272-2), as described previously. From each positive sample, one isolate was submitted to detection of *C. jejuni* or *C. coli*, as described in a previous section.

Statistical Analysis

All the data obtained from *Campylobacter* counts were log-transformed to perform the statistical analysis. During the first trial, the data taken before and after chilling from each replication were compared using one-way ANOVA. The quantitative data obtained from the second trial were submitted to ANOVA to evaluate the differences between samples with and without skin and the differences among the counts obtained from samples taken at different storage times. The frequency distribution, and in particular the rate in samples with high counts, was analyzed using the chi-square or exact Fisher's test. The general trend of the values during storage was analyzed using the Cox–Stuart trend test. To perform the statistical analyses, the data were run on GraphPad Prism software (GraphPad Software, San Diego, CA); a two-sided P-value <0.05 was considered significant.

RESULTS AND DISCUSSION

Prevalence of Campylobacter Spp. in the Broiler Flocks

The data obtained from the first trial showed the wide distribution of *Campylobacter* spp. in the broiler population in the area considered: the evaluation of the cecal content of the slaughtered animals showed the presence of the pathogen in all the 13 analyzed flocks of broilers that were reared in different farms but with similar farming protocols, thus suggesting the presence of similar contamination patterns. Previous data indicate a variable prevalence of cecum colonization, with a mean prevalence of 63.3% obtained from the baseline survey performed in the European Union and referred to Italy. In particular, considering the regions of Northern Italy where the animals were reared, lower frequencies were detected from previous studies as per traditional or biomolecular methods (56.3–82.9%) (Pezzotti et al., 2003; Di Giannatale et al., 2010; Robino et al., 2010). It has to be noted that, in the case of samples with high *Campylobacter* counts (as those occurred in cecal content), a good detection rate could be obtained using the count method, as stated in the improved version of the ISO 10272-1 method, which is currently applied just since 2017.

Counts ranging from 5 to 6 log cfu/g were constantly detected, without evident differences among the farms; these results were similar or lower than those detected in previous studies performed in Europe (Rosenquist et al., 2006; Seliwiorstow et al., 2015).

First Trial: Evaluation of On-Chain Chilling of Carcasses

During the first trial, the eventual decontaminating effect of on-chain carcass refrigeration was evaluated. Based on the prevalence data and taking into account the slaughtering techniques applied, a very high frequency of contaminated carcasses was expected, with a likely cross contamination among the flocks (contamination between separated carcasses and between different flocks via contaminated scalding water and defeathering equipment). Indeed, all the samples taken from the carcass skin both before and after the chilling step showed the presence of *Campylobacter* spp. with counts higher than 0.7 log cfu/g. This contamination seems to be unavoidable and was evidenced in previous studies

(Berrang et al., 2001; Seliwiorstow et al., 2015): data obtained from Italian slaughterhouses showed prevalence up to 80% of contaminated carcasses (Pezzotti et al., 2003; Manfreda et al., 2006; Pepe et al., 2009; Di Giannatale et al., 2010; Comin et al., 2014).

In this situation, attention should be paid mostly to the efforts to limit the number of bacteria present on the surfaces of skin and meat, rather than to completely prevent the presence within a flock; previous studies demonstrated the possibility of lowering the contamination level in carcasses even if positive flocks are slaughtered, which is dependent on the plant considered and on the procedures applied (Habib et al., 2012; Seliwiorstow et al., 2015).

The counts of *Campylobacter* spp. obtained from the analysis of the neck or breast skin before and after on-chain chilling are reported in Table 1. The data obtained showed a wide variability in the counts, with a range of 3 logs, both before and after chilling; such variability was mainly observed among the flocks, whereas a limited variability was often detected within each flock. Thus, a high correlation ($r = 0.81$) was found between the counts obtained before and after chilling. In addition, if cross contamination could occur during chilling, it appeared to be limited: it could be argued that strong ventilation could spread droplets during the initial chilling phase, when the carcasses are still wet after wash, but the distance among the carcasses should limit this spread.

As shown in Table 1, a slight but significant difference ($P = 0.011$, applying a statistical analysis to paired data) was observed between the samples taken before and after the air chilling phase, with a mean difference of about 0.3 log cfu/g. A reduction of almost 0.1 log was measured in 9 of 13 different flocks, suggesting that the on-chain chilling step could be considered as a low but useful hurdle in the control strategy applied by the FBO. The effect observed was coupled with a mean drop of 14.7°C of the skin surface temperature (from 21.3°C–6.6°C), whereas the subcutaneous temperature decreased by 9.1°C (from 24.4°C–15.3°C). Our data agree, both for the mean decrease obtained and for the variability, with those obtained by Seliwiorstow et al. (2015) when applying air chilling. Thus, the reduction rate appears not to depend strictly on the temperature reached on the carcass surface; this could be due to the fact that *Campylobacter* sp. survival (and not growth) was assessed. The influence of chilling was observed when considering the number of samples with high *Campylobacter* sp. counts (≥3 log cfu/g): the rate among samples taken after chilling (18/39) was indeed lower ($P = 0.010$) than that detected before chilling (30/39).

The counts obtained after carcass chilling (about 3 log cfu/g) were in accordance or slightly higher than those obtained by other studies (1.43–3.26 log cfu/g) (Rosenquist et al., 2006; Comin et al., 2014; Seliwiorstow et al., 2015); a similar picture was observed when considering the rate of high counts (46% from our data, with a range of 6–56% from the literature) (EFSA, 2010a; Habib et al., 2012; Comin et al., 2014; Seliwiorstow et al., 2015). It has to be noted that, in the case of our study, not all the chilling phases were assured by this on-chain step, which should be regarded as one of the complex series of hurdles.

Table 1. *Campylobacter* sp. counts obtained from the neck or breast skin of broiler carcasses before and after the on-chain chilling phase.

		Campylobacter sp. mean counts (SD), log cfu/g	
Replication	Batch no.	Before chilling	After chilling
1	1	3.05[a](0.11)	2.59[b](0.26)
	2	3.80 (0.40)	3.43 (0.23)
	3	4.31 (0.27)	4.30 (0.28)
	4	3.78 (0.06)	3.84 (0.26)
	5	3.23 (0.16)	3.11 (0.17)
2	6	4.22 (0.53)	4.33 (0.12)
	7	1.85 (0.31)	2.03 (0.13)
	8	3.39 (0.33)	3.26 (0.27)
	9	3.18 (0.22)	2.81 (0.23)
	10	3.74[a](0.30)	3.10[b](0.11)
3	11	2.52 (0.58)	2.00 (0.30)
	12	2.85 (0.28)	2.24 (0.46)
	13	3.47[a](0.41)	2.38[b](0.19)
Total		3.36[a](0.73)	3.03[b](0.81)
Minimum value		1.65	1.69
Median value		3.40	2.97
Maximum value		4.68	4.56

[a,b]Superscript letters on the same row indicate a statistically significant difference ($P < 0.05$) between the counts obtained before and after chilling.

Second Trial: Evaluation of Meat Chilling After Slaughtering

During the second trial, the effect of the second chilling phase, applied at the end of the slaughtering procedures, was evaluated, by taking a total of 63 broiler carcasses or cuts, with different storage times at 2°C to 3°C, to monitor the influence of the phase on *Campylobacter* sp. counts and the condition of meats that are ready for delivery to retailers. The distribution of the counts detected in the samples is shown in Table 2.

The counts obtained were lower than those obtained from chilled samples during the first trial, which was as expected: *Campylobacter* spp. were not detected in 9 samples (counts lower than 0.7 log cfu/g), and about 80% of the counts fell within the range of 1–3 log cfu/g, without significant differences between samples with and without skin, considering the whole sample population. These data were expected, as a general decrease in

Table 2. Distribution of *Campylobacter* sp. counts in the samples taken during postprocessing storage.

Sample typology		General	Skin-on	No skin
Median value (log cfu/g)		1.88	1.79	2.00
No. of samples	<1 log cfu/g	7	5	2
	1–2 log cfu/g	26	15	11
	2–3 log cfu/g	26	14	12
	>3 log cfu/g	4	2	2
	Total	63	36	27

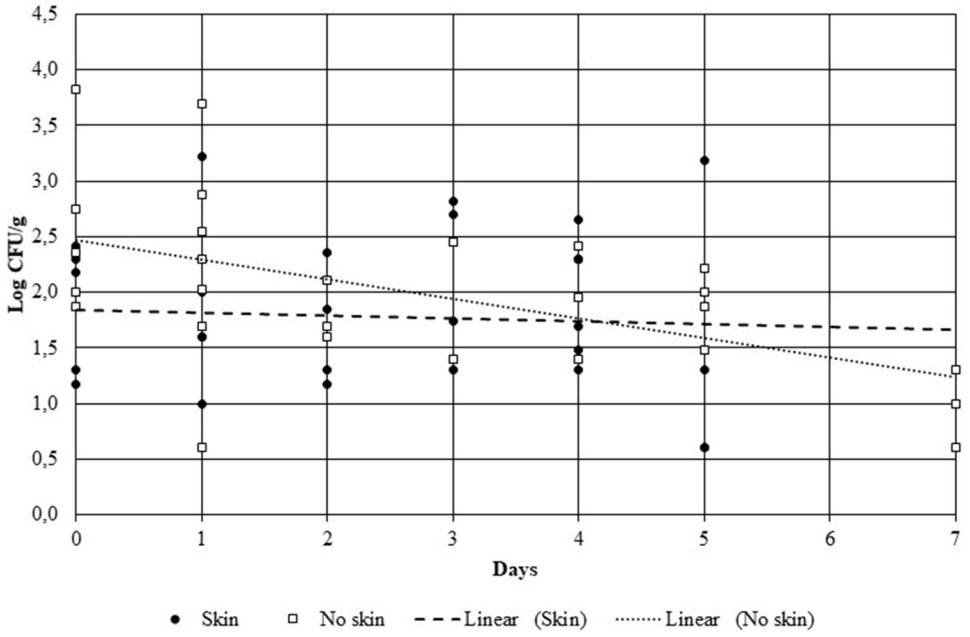

Figure 1. Distribution of *Campylobacter* sp. counts in the samples taken throughout the storage.

counts during the whole slaughtering process has been previously described, in particular during the scalding, washing, and cooling phases (Rosenquist et al., 2006; Allen et al., 2007; Berrang et al., 2007). A decreasing trend was observed in samples without skin ($P = 0.046$) as long as the samples were stored at chilling temperature (Figure 1), suggesting an impact of continuous chilling and storage on the *Campylobacter* survival rate, rather than a drop in the counts due to a single chilling phase. However, to better demonstrate the effect of cooling on *Campylobacter* sp. counts, the data were analyzed by considering the counts obtained from samples stored for 0 to 1 d, 2 to 4 d, and more than 4 d. High counts ($\geq$3 log cfu/g) were observed mainly (also if not significantly) in samples taken on day 0 to 1 of storage (3 of 25 samples), whereas only one sample (of 38 samples) taken after longer storage time, showed high values.

A different trend was observed between the samples with or without skin throughout the storage period (Figures 2A and 2B): no evident decrease in the count distribution was in fact observed in the skin-on samples. Alternatively, the samples without skin showed a marked decrease, with an evident difference ($P = 0.030$) between samples taken at day 0 to 1 and those taken at day 4 or more. Theoretical decrease curves were built, based on the distribution of the counts throughout the whole storage period considered (Figure 1) to be calculated, estimating a decrease of 0.03 log cfu/g per day in the skin-on samples and of 0.18 log cfu/g per day in samples without skin. Our data are in agreement with those obtained by other authors (Chantarapanont et al., 2003; Davis and Conner, 2007), which suggests the probability of a higher survival rate for bacteria housed within the crevices and feather follicles, in part due to the inability of water to reach bacteria for removal and the decrease in oxygen present, creating a microaerobic atmosphere. The mechanism involved is not fully understood.

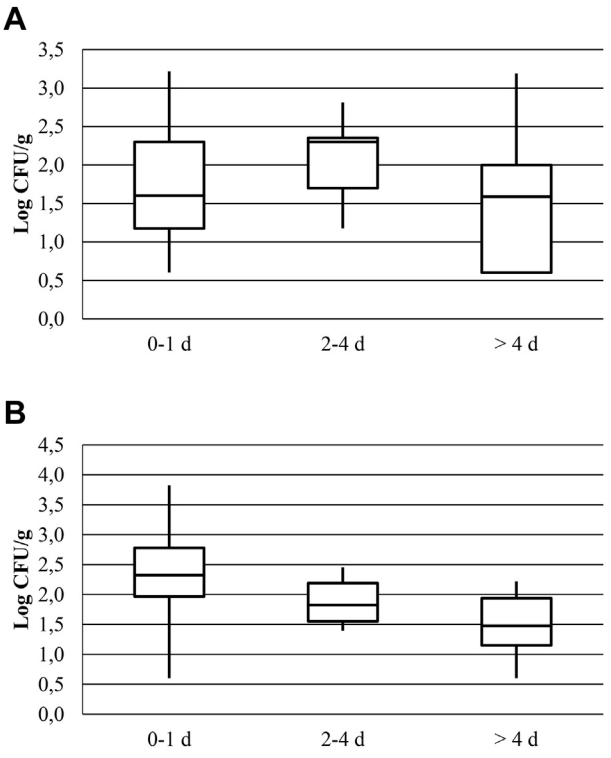

Figure 2. Distribution of *Campylobacter* sp. counts on carcass or cut samples (A) with skin or (B) without skin during the chilled storage.

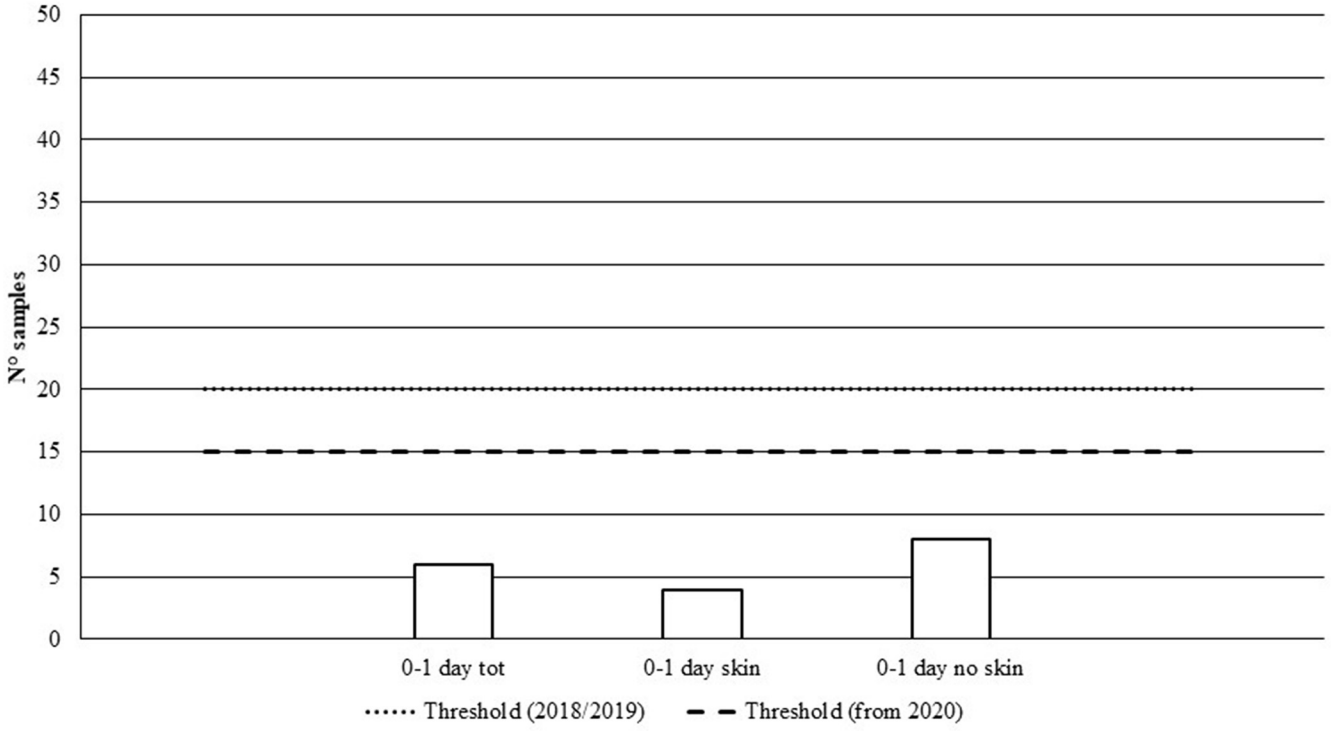

Figure 3. Comparison of the data obtained on the chilled broiler meat samples with the tolerance threshold set by EC Regulation 2073/2005.

Campylobacter Species Distribution

A total of 75 isolates were identified as *Campylobacter* spp. by PCR; all these isolates were identified by PCR as *C. jejuni* or *C. coli*, with no other species being identified. The most prevalent species was *C. jejuni* (72% of the isolates); no evident differences were detected from the comparison of samples taken after different storage times or samples with and without skin.

The prevalence of *C. jejuni* observed in this study was not observed in previous studies performed in Northern Italy (Di Giannatale et al., 2010), but variable relative rates for the 2 species have been described in Italy by other authors (Manfreda et al., 2006; Pepe et al., 2009; EFSA, 2010a; Di Giannatale et al., 2014).

Requirements of EC Regulation 2073/2005 and Needs of the FBO

The data obtained from this study must be considered in the light of the current law requirements that are intended mainly to limit the delivery of meat samples with high counts. This approach derives from a balance of the need for consumer protection and the feasibility in the current slaughtering processes. As per EFSA (2011), the current limit of 1,000 cfu/g on the neck or breast skin should reduce the health risk by more than 50%, while a noncompliance rate of about 15% of the flocks was foreseen. In the current situation of the plant where the study was performed, the official sampling by the FBO was performed after the first on-chain chilling step: thus, the results obtained show an "unacceptable" result, based on the definition of the Regulation, even considering the "old" (20/50) or the "new" (15/50) tolerance threshold.

Thus, improvements by the slaughtering process are needed; the use of an empowered chilling tunnel could exert some positive effect mainly on the total microbial population (including widespread microorganisms, i.e., e.g., *Enterobacteriaceae* and psychotropic *Pseudomonas* spp.), but in the light of the data obtained from the study and from the literature, this could have little impact on *Campylobacter* counts. A lower presence of residual feathers on the carcass after defeathering could hopefully favor the decontamination effect of the further washing and chilling steps; on the other hand, a higher intensity of defeathering would impact negatively on the escape of fecal material from the cloaca (Berrang et al., 2001, 2018). A general evaluation of the situation of other plants is still difficult: the data from other studies (Comin et al., 2014; Seliwiorstow et al., 2015) show similar pictures, but the official data supplied by the EFSA reports are limited, as only results from 8 member states, from the year 2018, are available (EFSA, 2019), with a mean value of 18.4% of samples exceeding the 1,000 cfu/g limit, but with a wide variability among the countries (0–100%).

To fulfill the requirements of the Regulation, the FBO also need to set sampling conditions, that is, the sampling point throughout the production process. EC Regulation 2073/2005 states that sampling must be performed "after chilling," but the definition of the end of the chilling process should be clearly defined. As shown in Figure 3, if we consider that the "chilling" phase is completed when the carcass temperature reaches 4°C (required by EU Regulation No. 853/2004; European

Community, 2004), the data obtained from the analysis of carcasses and cuts at day 0 to 1 from slaughtering could be chosen, leading to a "satisfactory" result, without the need for improvements in the process. This choice should be made in agreement with the competent authority, whose role has been enhanced by the enforcement of EU Regulation No. 627/2019 (EU [European Union], 2019). The new Regulation ensures that official controls are made by the competent authority repeating samples at broiler slaughterhouses (at least 49 per year for each industrial plant), applying the same methods as the FBO (currently performed for *Salmonella* spp.), with the aim to warrant the reliability of routine self-check.

CONCLUSIONS

The distribution of *Campylobacter* spp. within the broiler chicken population and on meat in Northern Italy is currently very high, and the options available to reduce it during the slaughtering process are limited. This is due to the requirements of the legislation in force (as decontaminating agents are not allowed in the carcass washing phase) and to the market demand for fresh (not frozen) broiler meat with a long residual shelf life to be supplied to the large-scale retailers. In addition, a slight decontaminating effect could be exerted by every single step, and chilling could be considered as a useful intervention included by the FBO in a general food safety management system. This approach would favor both the fluent functionality of the production line and the supply of meats with low risk of *Campylobacter* contamination by the combined application of several mild hurdles.

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DISCLOSURES

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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