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Detection of *Campylobacter* spp. and hygiene indicators along the poultry slaughter line

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ABSTRACT

Campylobacter spp., a leading cause of foodborne disease, is closely associated with poultry meat. The slaughter line process involves numerous steps, which can contribute to cross-contamination with microorganisms. Our study aimed to assess the hygiene of the poultry slaughter process by determining levels of *Campylobacter* spp. and other relevant bacterial indicators of fecal contamination. Research was conducted in a medium-capacity poultry slaughter facility where most steps are automated. Sampling included broilers from two farms. Neck skin samples were collected for *Campylobacter* spp. analysis after both the defeathering and cooling processes. Additionally, swab samples for microbiological examination were taken from surfaces of both the defeathering machine and a meat-cutting table. Standard ISO methods were followed for quantitative microbiological analysis. The findings of *Campylobacter* spp. in neck skin and on surfaces that contact the carcasses were confirmed by PCR. Our findings reveal a strong correlation between the *Campylobacter* spp. counts on the neck skin and the levels of this pathogen detected on the tested surfaces. Furthermore, the aerobic bacteria count on the surfaces corresponds to both the *Enterobacteriaceae* count and the *Escherichia coli* count. A high degree of contamination with *Campylobacter* spp. (mean count in neck skin after cooling >3 $\log_{10}$ CFU/cm^2) and fecal contaminants (*Enterobacteriaceae* and *E. coli*) was detected in the examined poultry slaughterhouse. Therefore, the rules of good hygiene practice and hazard analysis and critical control point (HACCP) principles need to be reinforced in the facility with the aim of improving slaughter hygiene and product safety. The food business operator should review their food safety system, implement stricter hygiene measures in the facility, check the suppliers (farms and carriers) and apply good hygiene practices and biosecurity measures.

1. Introduction

In the European Union, according to data from the European Food Safety Agency (*EFSA*, 2023), campylobacteriosis is the most frequently registered foodborne disease, while according to data from the Institute for Public Health of Serbia, this disease is in second place in our country after salmonellosis (*IJZS*, 2020). Numerous vectors can contribute to the spread of *Campylobacter* spp. in the food chain, and one of the key ones is poultry meat (*EFSA* and *ECDC*, 2023). The process of slaughtering and processing poultry is complex and includes many process steps, which also affects the possibility of contamination and spread of bacteria during operations. To ensure appropriate process hygiene and product safety, strict adherence to good hygiene practices along with control measures based on the assessed risk or hazard analysis and critical control points (HACCP) principles is expected (*Althaus et al*., 2017). One of the key parameters for assessing the effectiveness of hygiene operations at slaughter is whether there is an increase or decrease in the number of microorganisms throughout the

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production process. Considering that poultry meat is often contaminated with *Campylobacter* spp., regulatory bodies have established a microbiological criterion (1000 CFU/g) for assessing the hygiene process of poultry slaughter (*EC*, 2005; *Serbia*, 2010). The objectives of this study were to examine the hygiene process of poultry slaughter by monitoring the number of *Campylobacter* spp., examining selected microbiological indicators (number of aerobic bacteria, number of *Enterobacteriaceae*, and number of *Escherichia coli*), and observing whether the number of tested bacteria shows a decreasing trend.

2. Materials and Methods

2.1. The Slaughterhouse and Slaughter Operations

The research was conducted in December 2023 at a medium-capacity poultry slaughterhouse (4000 broilers h^{-1}) where most of the processing steps are automated. After unloading, broilers were hung/shackled and then stunned with electric current, followed by automatic bleeding. Scalding was performed by immersion in a water tank at 50±1 °C, and was followed by automatic feather removal. After evisceration (manual) and final carcass processing, the carcasses were washed with cold water to remove visual contamination before cooling. The cooling process took place in a chamber at −1 °C to 0 °C with appropriate air circulation. The cooling process lasted for 3 h until the temperature in the carcass deep muscle reached ≤4 °C. Hygiene assessment at the slaughterhouse was carried out in accordance with standard operating procedures. The cleanliness assessment of the slaughter line was conducted using a scale (1 – clean, 2 – soiled, 3 – dirty) before the start of operations. The cleanliness of the slaughter line was rated as 1 before the start of operations. After completing the broiler slaughter from farm A, the slaughter line was thoroughly cleaned and washed before starting the broiler slaughter operations from farm B.

2.2 Farms of Origin and Transport

Broilers originated from two farms (farm A and farm B). The broilers were transported to the slaughterhouse using appropriate vehicles, with each cage containing 10 broilers. The total number of broilers transported from farm A was 2500, while 1600 were transported from farm B. The transport from farm A to the slaughterhouse took 30 minutes, and from farm B it took 90 minutes. The outside temperature at the slaughterhouse location was 2±1 °C with a relative humidity of 65%. There were no mortalities or injuries after transport. The age of the broilers was over 42 days (farm A – 48 days old; farm B – 49 days old). The mean broiler weight of live animals from farm A was 3.08 kg, and from farm B was 2.70 kg. The duration from arrival of the shipment at the slaughterhouse to the start of slaughter operations was 5 h. After unloading, in accordance with good hygienic practice, the transport vehicles were cleaned, washed and disinfected.

2.2. Sampling and Samples for Testing

Sampling for microbiological testing was conducted 30 min after the start of the slaughter operations. For the examination of the number of *Campylobacter* spp., neck skin samples were taken after the plucking/defeathering and cooling phases from a total of 36 slaughtered broilers (farm A – 18 carcasses and farm B – 18 carcasses). At each sampling point, 10 g of neck skin was taken from 9 carcasses, forming three composite samples (3×30 g). For microbiological testing of surfaces (*Campylobacter* spp., number of *Enterobacteriaceae*, number of aerobic bacteria, number of *E. coli*), a total of eight samples were taken (farm A – 4 swabs and farm B – 4 swabs). From each farm, two swabs were taken from the surfaces of the plucking machine and the carcass cutting table. The swab samples were taken from an area of 100 cm^2 that was in direct contact with the carcass surface, using the standard method (*Serbia*, 2018).

2.3. Microbiological Testing

The determination of the *Campylobacter* spp. count on broiler neck skin was performed using the standard method (*Serbia*, 2023). Briefly, each composite sample (3×10 g) was individually minced, and for the determination of *Campylobacter* spp., 10 g of the minced neck skin was homogenized with 90 mL of maximum recovery diluent (MRD, Oxoid). Further decimal dilutions were made from the primary dilution and inoculated onto appropriate microbiological media in accordance with the standard testing method (*Serbia*, 2023). For microbiological testing of swabs, 10 mL of MRD was added to each swab, from which further decimal

dilutions were made and inoculated onto appropriate microbiological media (Oxoid) in accordance with standard methods: *Campylobacter* spp., *Serbia* (2023); *Enterobacteriaceae* count, *Serbia* (2017); aerobic colony count, *Serbia* (2022); *E. coli* count, *Serbia* (2008).

2.4. Molecular Testing

From each plate, suspect colonies of *Campylobacter* spp. were transferred into a 1.5 mL tube containing 100 µL of phosphate buffer. After brief vortexing for 10 s, the tubes were placed in a thermal mixer with blocks (Thermo Fisher Scientific, USA) for complete microbial inactivation at 95 °C for 5 min. DNA isolation was conducted using a commercial kit for genomic DNA purification (GeneJET Genomic DNA Purification Kit*,* Thermo Fisher Scientific, USA). Polymerase chain reaction (PCR) was performed in a final volume of 25 µL, containing the following components: DreamTaq PCR Master Mix (2X) (Thermo Fisher Scientific, USA), 500 nM of each primer (Table 1) and 0.5 µL of isolated DNA. The PCR process involved initial denaturation for 5 min at 95 °C, followed by 40 cycles: 45 s at 94 °C, 45 s at 51 °C, and a final extension for 5 min at 72 °C. Electrophoresis of PCR products was conducted on a 2% agarose gel, with Midori Green Advance dye (Nippon Genetics, Japan), at 95 V for 45 min.

2.5. Statistical Analysis

Before any statistical analysis, the obtained data were tested for normality using the Shapiro-Wilk test ($p > 0.05$). Differences in microbiological parameters at the slaughter line between the two broiler origin farms were examined using an independent *t*-test. The estimation of correlation between the tested microbiological parameters was conducted using Pearson's correlation test. All values are expressed as mean±standard error. Statistical analysis of the results was performed using the SPSS 23 software package (SPSS Inc., Chicago, IL, USA).

3. Results and Discussion

The mean *Campylobacter* spp. counts in the neck skin of broilers along the slaughter line are presented in Table 2. In the neck skin samples taken immediately after the plucking phase, the number of *Campylobacter* spp. on broilers originating from farm A was significantly higher than the number on broilers originating from farm B. The reduction in *Campylobacter* spp. (log reduction from after plucking to after cooling) was 0.95±0.02 $\log_{10}$ CFU/g in the examined broilers from farm A, while this reduction was lower in broilers from farm B (Table 2). Although a reduction in the number of *Campylobacter* spp. was achieved during the cooling phase, as expected, due to the initially high number of these bacteria after the feather plucking phase, the number of these bacteria remained high and exceeded the critical limit (1000 CFU/g or 3.0 $\log_{10}$ CFU/g). According to *Althaus et al.* (2017), 29% of the examined poultry carcasses tested positive for *Campylobacter* spp., of which 42% had a bacterial count >3.0 $\log_{10}$ CFU/g. These authors found that the number of *Campylobacter* spp. decreases during the carcass scalding phase, but on average, the number of these bacteria increases (by 0.4 $\log_{10}$ CFU/g) after the plucking/defeathering phase. The risk of consumer infection increases with a higher number of bacteria contaminating the meat or product, emphasizing the importance of monitoring *Campylobacter* spp. in poultry meat production facilities. According to the current regulations in Serbia, out of 50 examined composite samples of poultry neck skin after cooling, no more than 15 examined composite samples should exceed the established limit (*Serbia*, 2010). Considering the importance of *Campylobacter* spp. for public health, this criterion will be tightened from 2025, and out of 50 examined samples, no more than 10 examined samples will be allowed to exceed the established threshold. If a food business operator (FBO) fails to meet the required criterion, the risk to public health increases, and it is the obligation of the FBO to review its system and implement corrective measures to verify and validate its food safety management procedures and good hygiene practices (*EFSA*, 2023).

Table 1. Primers used for the detection of the 16S rRNA region of *Campylobacter* spp.

The name of the primer	Sequence (5'–3')	Product size	Authors
16 S rRNA – F	ATCTAATGGCTTAACCATTAAAC	856 bp	*Dennis et al*., 1999
16 S rRNA – R	GGACGGTAACTAGTTTAGTAT		

Table 2. *Campylobacter* spp. counts (log_{10} CFU/g; mean±standard error) in the neck skin of broilers sampled on the slaughter line

	Farm A	Farm B	P-value
After plucking/defeathering	4.47±0.01	3.67±0.03	0.001
After cooling	3.52±0.03	3.12±0.12	0.05
Log reduction	0.95±0.02	0.56±0.1	0.08

In addition to examining the presence and count of *Campylobacter* spp. on the neck skin of broilers, the number of these microorganisms was also examined on surfaces that come into contact with the carcass/meat. By amplifying the 16S rRNA-specific region, the presence of *Campylobacter* spp. genomes was determined (Figure 1). Swab samples were taken from the surfaces of the defeathering machine and the carcass cutting table. The number of *Campylobacter* spp. on the examined surfaces of the feather plucking machine was significantly higher during the processing operations of broilers from farm A compared to farm B (Table 3). Considering that broilers from farm A had a significantly higher number of *Campylobacter* spp. in the examined neck skin samples (Table 2), greater contamination of the feather plucking machine surfaces during the processing of this group of animals is expected. No significant difference was found in the *Campylobacter* spp. count from the carcass cutting table during the slaughter of broilers from farm A and farm B.

Due to the complexity of operations and the relatively high level of automation in the poultry slaughter line, a large number of surfaces become contaminated during the process. *Campylobacter* spp. have the ability to form biofilms (*Laconi et al.*, 2023), and these can be a constant source of cross-contamination on the slaughter line. Biofilm forms after bacteria adhere to a surface and is significantly more difficult to remove than planktonic bacteria, so regular hygiene maintenance and constant removal of organic matter from the slaughter and processing line are of great importance, including washing and disinfection (*Araújo et al.*, 2022).

In addition to determining the presence of pathogenic microorganisms, the meat industry also monitors process hygiene indicators. For these investigations, the selected indicators are the numbers of aerobic bacteria, *Enterobacteriaceae* and *E. coli.* The number of aerobic bacteria generally indicates the number of bacteria and hygiene, while *Enterobacteriaceae* and *E. coli* are indicators of fecal contamination on the slaughter line (*Althaus et al.,* 2017). The number of aerobic bacteria found in surface swabs is shown in Table 4. A significantly higher number was found on the surfaces of the feather plucking machine during the slaughter phase of broilers from farm A compared to farm B. Significantly higher numbers of *Enterobacteriaceae* (Table 5) and *E. coli* (Table 6) were determined in surface swabs during the slaughter of broilers from farm A than of broilers from farm B, both on the surfaces of

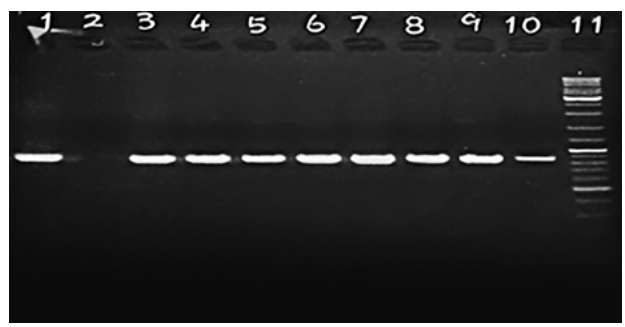

Figure 1. *Campylobacter* 16sRNA PCR test on agarose gel: (1) Positive control — *Campylobacter jejuni* ATCC 33560; (2) Negative control — water; (3–6) Samples from neck skin; (7–10) Swab samples; (11) DNA marker

Table 3. *Campylobacter* spp. counts (log_{10} CFU/g; mean±standard error) in swab samples from surfaces during the slaughter of broilers originating from different farms

	Farm A	Farm B	P-value
Plucking/defeathering machine	1.99±0.16	1.17±0.09	0.01
Meat-cutting table	0.52±0.13	0.84±0.11	0.16

Table 4. Aerobic bacteria counts (log_{10} CFU/cm^2; mean±standard error) in swab samples from surfaces during the slaughter of broilers originating from different farms

	Farm A	Farm B	P-value
Plucking/de-feathering machine	4.70±0.06	2.84±0.20	0.001
Meat-cutting table	3.93±0.04	3.42±0.08	0.006

Table 5. *Enterobacteriaceae* counts (log_{10} CFU/cm^2: mean±standard error) in swab samples from surfaces during the slaughter of broilers originating from different farms

	Farm A	Farm B	P-value
Plucking/de-feathering machine	2.08±0.18	0.84±0.18	0.009
Meat-cutting table	1.22±0.14	0.74±0.07	0.04

Table 6. *Escherichia coli* counts (log_{10} CFU/cm^2: mean±standard error) in swab samples from surfaces during the slaughter of broilers originating from different farms

	Farm A	Farm B	P-value
Plucking/de-feathering machine	1.48±0.26	0.30±0.16	0.02
Meat-cutting table	0.79±0.11	0.38±0.05	0.03

the feather/plucking machine and the carcass/meat cutting table.

Correlation analysis of the parameters investigated on the broiler slaughter line (Table 7) showed a high degree of correlation between the *Campylobacter* spp. count on the neck skin and the number of these bacteria on the slaughter line surfaces. Also, on the examined slaughter line surfaces, a high degree of correlation was measured between the aerobic bacteria count and the *Enterobacteriaceae* count. This correlation trend was observed in the case of the *E. coli* count, which was also directly correlated to the *Enterobacteriaceae* count on the slaughter line surfaces (Table 7).

Table 7. Correlation analysis of the parameters examined on broiler contact surfaces in the broiler slaughter line

	***Campylobacter* spp. count in neck skin**	***Campylobacter* spp. count from surface swab**	**Aerobic bacteria count from surface swab**	***Enterobacteriaceae* count from surface swab**
Escherichia coli count from surface swab	0.73**	0.63*	0.78**	0.96**
Enterobacteriaceae count from surface swab	0.85**	0.70*	0.78**	
Aerobic bacteria count from surface swab	0.64*	0.43		
Campylobacter spp. count from surface swab	0.83**			

* $p < 0.05$; ** $p < 0.01$

4. Conclusion

In this study, quantitative microbiological analysis was conducted of *Campylobacter* spp. on broiler carcasses (neck skin) and on selected surfaces in contact with the broiler carcass/meat. *Campylobacter* spp. were detected on the neck skin of the examined carcasses after both feather removal and carcass cooling, with the determined mean counts being >3 $\log_{10}$ CFU/g. Slaughter line surfaces were contaminated with both *Campylobacter* spp. and fecal indicator bacteria (*Enterobacteriaceae* and *E. coli*). In accordance with good hygiene practices and HACCP principles, in slaughter facilities where unacceptable bacterial contamination is measured, FBOs should reassess their food safety systems and implement enhanced hygiene measures in the facility, including supplier (farm) checks and adherence to good hygiene practices and biosecurity measures within the system. The aim is to reduce the presence of both *Campylobacter* spp. and bacterial indicators of process hygiene on slaughtered broiler carcasses.

Nalaz *Campylobacter spp.* i indikatora higijene na liniji klanja živine

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INFORMACIJE O RADU

Ključne reči:
Higijena
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Hrana
Proces

APSTRAKT

Campylobacter spp. su vodeći uzročnici bolesti prenosivih hranom, a jedan od ključnih vektora je meso živine. Operacije klanja uključuju veliki broj procesnih koraka, što utiče i na mogućnost unakrsne kontaminacije mikroorganizmima. Naša studija ima za cilj da ispita higijenu procesa klanja živine putem praćenja broja *Campylobacter* spp. kao i ostalih odabranih mikrobioloških indikatora higijene procesa. Istraživanje je sprovedeno u objektu za klanje živine srednjeg kapaciteta, gde je većina procesnih koraka automatizovana. Uzorkovanjem su obuhvaćeni brojleri poreklom sa dve farme. Za ispitivanje broja *Campylobacter* spp. uzeti su uzorci kože vrata, nakon čerupanja i hlađenja. Za mikrobiološko ispitivanje površina *(Campylobacter* spp., broj enterobakterija, broj aerobnih kolonija, broj *E. coli*), uzeti su uzorci brisa sa površina mašine za čerupanje perja i stola za rasecanje mesa. Kvantitativna mikrobiološka analiza sprovedena je standardnim SRPS ISO metodama. Nalaz *Campylobacter* spp. na uzorcima kože vrata i brisevima površina potvrđen je PCR tehnikom. Može se konstatovati visok stepen korelacije nalaza broja *Campylobacter* spp. sa kože vrata i broja ovih bakterija sa ispitivanih uzoraka površina. Takođe, sa porastom nalaza broja aerobnih kolonija u ispitivanim uzorcima površina, može se primetiti i visok stepen korelacije porasta broja enterobakterija koji je u direktnoj vezi sa brojem *E. coli*. S obzirom da je u ispitivanom objektu za klanje živine, utvrđen visok stepen kontaminacije *Campylobacter* spp. i fekalnim kontaminentima (enterobakterije i *E. coli*) na trupovima i površinama, u skladu sa pravilima dobre higijenske prakse i principima HACCP, subjekat u poslovanju hranom treba da preispita sistem bezbednosti hrane i primeni pooštrene mere higijene pogona, uz proveru dobavljača (farme i prevoznici) i primenu principa dobre higijenske prakse i biosigurnosnih mera u sistemu sa ciljem unapređenja higijene klanja i bezbednosti proizvoda.

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