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THE OCCURRENCE OF *SALMONELLA* SPP. AND *CAMPYLOBACTER* SPP. IN BROILER CHICKEN CARCASSES FROM ONE COMMERCIAL POULTRY PROCESSING PLANT IN SERBIA

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ABSTRACT

The aim of this study was to determine the occurrence of *Salmonella* spp. and *Campylobacter* spp. in broiler chicken carcasses in one poultry slaughterhouse in Serbia during a two-year period. The research was conducted to check the hygiene of the production process. In total of 360 random samples were collected during the 2022 and 2023, through 24 individual sampling. It was found that 84 samples (23.33%) were contaminated with *Salmonella*, with a higher rate of contamination observed during spring and summer (69 isolates, 82.1%). *Salmonella Enteritidis* was the predominant serovar, followed by *Salmonella Infantis* and *Salmonella* spp. gr C1. In the first year of sampling, the mean number of *Campylobacter* spp. was 613.4 CFU/g, whereas in the second year the contamination with these bacteria increased and amounted to 1169.17 CFU/g. There was a significant seasonal variation in the level of contamination of broiler chicken carcasses with *Campylobacter* spp, with the highest number recorded in the summer months. In conclusion, carcass contamination by *Salmonella* and *Campylobacter* in broiler chickens is a significant public health issue in Serbia and and requires continuous monitoring and implementation of preventive control procedures.

Keywords: *Salmonella, Campylobacter*, poultry carcasses, process hygiene criteria

INTRODUCTION

Campylobacteriosis and *Salmonellosis* are two of the most significant emerging food-borne zoonotic diseases in the world, despite the application of numerous eradication measures. According to EFSA (European Food Safety Authority) latest reports, in 2022, these were the first (137.107 cases) and second (65.208 cases) most reported zoonoses in humans (**EFSA, 2022**). They can be found in the gastrointestinal tract of various animal species, either as commensals or as pathogens. In addition to numerous potential transmission vehicles, raw and undercooked poultry meat has been identified as one of the most important (**Furukawa *et al*., 2017**). All stages in poultry meat production, from primary production at rearing farms, to the handling and consumption of chicken meat products at home, have a role in the transmission of these bacterias (**Skarp *et al*., 2016**). The most critical operations during poultry slaughtering are: skinning, plucking of feathers, evisceration, washing, and cooling (**Shung *et al.*, 2022**). Thermophilic *Campylobacter* spp. are zoonotic Gram-negative, non-spore forming bacterial pathogen, frequently associated with human acute gastroenteritis. There are three Campylobacter species: *C. jejuni, C. coli,* and *C. lari* and all of them are commonly associated with poultry meat. Campylobacteriosis, in the majority of cases, is caused by *Campylobacter jejuni*, which is particularly adapted to poultry and constitutes their largest reservoir (**Jovanović *et al.*, 2020**). The digestive tract of poultry, with its low oxygen level and higher temperature (42 °C), represents an ideal environment for the growth of this thermophilic bacterium, spreads easily among poultry, leading to rapid colonization but without causing harm to this host. On the other hand, it represents a primary source of exposure and plays a major role in causing gastroenteritis in humans (**Sadek *et al.*, 2023**). The survival of Campylobacter species mainly depends on the environmental conditions in which they grow, including temperature, so there is a noticeable seasonal variation in the number of people infected with these bacteria throughout the year (**Al-Qadiri *et al.*, 2015**). *Salmonella* zoonotic serotypes are a significant biological issue that seriously puts at risk the public's health. It Is the second most prevalent zoonotic agent in Europe, highly associated with poultry sector, specifically meat and eggs (**EFSA/ECDC, 2018**). According to the World Health Organization, there are over 2500 serotypes of *Salmonella*, but only, about 100 of them can be transmitted by food. Salmonella serovars *Typhimurium* and *Enteritidis* have been recognized as the major causes of *Salmonella* infections, but in recent years, serogroups C1 and C2 have also been frequently mentioned (**Fuche *et al*., 2018**). Due to cross-contamination, stress tolerance, and other characteristic, it is very hard to irradicate these bacteria in processing plants. Cross-contamination most often occurs due to contact of broiler meat with contaminated equipment (**Corry *et al.*, 2002**). Meat safety with respect to these two risks is ensured at the poultry chain's harvest level by preventing or minimizing the pathogens' transfer, i.e., abattoir process hygiene (**Buncic *et al*., 2017**). The Regulation (**EC No. 2017/1495**) on the control of *Salmonella* and *Campylobacter* ensures the implementation of adequate and appropriate measures taken to identify these pathogens at all stages of production, processing, and trade, in order to reduce the risk to public health. Due to the significant effects that they have on the economy and public health, a comprehensive effective risk mitigation strategy is necessary. A regulatory limit (microbiological process hygiene criterion (PHC) of *Campylobacter* on the neck skins of chilled poultry carcases is under 1000 CFU/g (**EC No. 2017/1495**).
Therefore, the objective of this study was to determine the level of hygiene in the production of broiler carcasses in a slaughterhouse based on occurrence of *Salmonella* spp. and *Campylobacter* spp. in one commercial poultry processing plant in accordance with the conditions defined by Commission Regulation (EC) No 2017/1495 and Rulebook on general and special hygiene conditions at any stage of production, processing and circulation (**"Official Gazzete of the RS", No. 72/2010 & 62/2018**). The second aim was to examine the seasonal influence on the appearance of these bacteria, as well as whether these bacteria are interdependent.

MATERIALS AND METHODS

Sample Collection

Sampling was carried out in a registered facility of medium capacity for the production and processing of poultry meat located in Sabac, Mačva district, in the western part of Serbia. Broilers processed in abattoirs originated from different farms, were raised for commercial purposes, and were slaughtered at 6 to 7 weeks of age. In total, 360 samples were collected during the 2022 and 2023, through 24 individual samplings. The sampling took place weekly, with the sampling day changing each week. After processing, washing, postmortem examination, and cooling were finished, 15 broiler chicken carcasses were randomly selected directly on the slaughter line. A piece of neck skin (10 g) was cut from each broiler chicken carcass to create a pooled sample using the method prescribed in the Annex to the Rulebook (**Official Gazette of the RS, 72/10&62/2018**). Pooled neck skin sample was made from three carcasses to form final samples of 5 × 25 g. The samples (three pooled neck skins) were packed in a sterile stomacher bag and transported to a laboratory in a manual refrigerator (0-4°C), within 3 h. Microbiological analysis were performed on the same day.

Microbiological Analysis

After manually homogenizing each pooled sample for one minute, the surplus weight from each of the three neck skins was equally eliminated, getting the final weight down to 25 g. The detection of *Salmonella* isolates was performed according to the **ISO 6579–1:2017**. For *Salmonella* isolation, 25g of broiler neck skin was mixed with 225 ml of buffered peptone water and incubated for 18 to 24 h at 37°C. Then, 1 ml was transferred to 10 ml of Muller Kauffmann tetrathionate-novobiocin broth (MKTTn, Oxoid, UK) and 0.1 ml was transferred to 10 ml of Rappaport Vassiliadis Broth with soya (RVS, Oxoid) and incubated at 37°C and 41.5 °C respectively, for 24 h. Cultures obtained from MKTTn and RVB broth were inoculated on two Petri plates with a selective medium Xylose Lysine Deoxycholate Agar (XLDA, Oxoid) and on Brilliant Green Agar plates (BGA, Oxoid). All the plates were incubated at 37°C for 24 h. Typical *Salmonella* colonies on XLDA grow in the form of red colonies with a black center, while on BGA they are pink. After incubation, a maximum of 5 colonies suspected of being *Salmonella* were selected for identification. For further confirmation of isolates, Salmonella-positive colonies were subjected to API 20E system (BioMerieux SA) and the Salmonella latex agglutination kit (Oxoid Ltd., Cambridge, United Kingdom). The detection of *Campylobacter* isolates was performed according to the **ISO 10272-1:2017**. From suitable dilutions, 0.1 ml volumes were placed onto Charcoal Cefoperozone Deoxycholate agar (CCDA, Oxoid) plates with special selective supplement. Following a 44-hour incubation period at 41.5 °C in a microaerophilic environment, typical colonies were determined on the plates. Campylobacter isolates were confirmed by oxidase and catalase tests, and *Campylobacter jejuni* was further differentiated by the hippurate hydrolysis test. Colony counts of quantitatively analyzed samples were expressed as CFU/g and the detection limit for Campylobacter was 10 CFU/g.

Statistical analysis

Statistical analysis of the results was elaborated using software GrapfPad Prism version 5.00 for Windows, GrapfPad Software, San Diego, California USA, www.graphpad.com i MS Excel. Descriptive statistics were used to express the mean log, standard deviation and coefficient of variation. Data were analysed statistically using the non-parametric test of Kruskal-Wallis for multiple comparisons. Fisher's exact test was used to determine the magnitude of the statistical difference between years. The statistical test assessed whether season exerted a significant effect ($P<0.01$) on the microbiological parameters. Microbial counts obtained in the collected samples is expressed as CFU/g.

RESULTS AND DISCUSSION

In this research, 360 broiler chcken carcasses were examined for the presence of *Salmonella* spp. and *Campylobacter* spp. It was found that 84 samples (23.33%) of the broiler meat were contaminated with *Salmonella* spp. *Salmonella Enteritidis* was isolated in 57 samples (67%), *Salmonella Infantis* in 15 samples (17%), whereas *Salmonella spp. gr C1* was detected in the remaining 12 samples (14%) (Figure 1). During the first year of sampling (2022), Salmonella was found in 60 samples (71.4%), and a statistically significant ($P<0.0001$) lower number of samples, only 24 positive samples (28.6%) were obtained during the second year of sampling (2023).

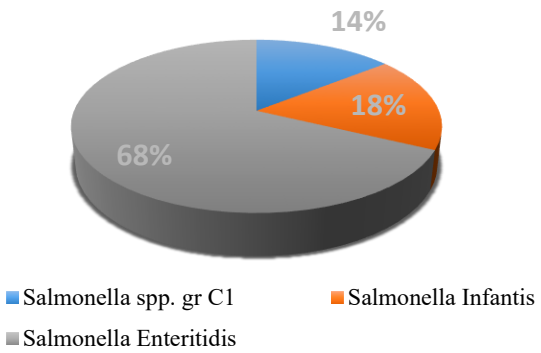

Figure 1 *Salmonella* serotypes identified in broiler carcass in 2022–2023

In total, 306 of 360 (85%) broiler chicken carcasses showed *Campylobacter* counts above the detection limit (>10 CFU/g). From these positive samples, in 84 (27.45%), the counts of *Campylobacter* was higher than the allowed determined by the current EU regulation (>1000 CFU/g). The proportion of samples containing more than 1000 CFU/g was 29.4% in 2022, and 70.6% in 2023, respectively, which is a statistically significant difference ($P<0.0001$).

The occurrence of *Campylobacter* in broiler chicken carcasses in every month, during the entire research period is presented in Figure 2. The highest average number of *Campylobacter* was isolated from the neck skin of broiler chicken carcasses sampled during the month of July, during both years of the study (1480 CFU/g; 5180 CFU/g, respectively).

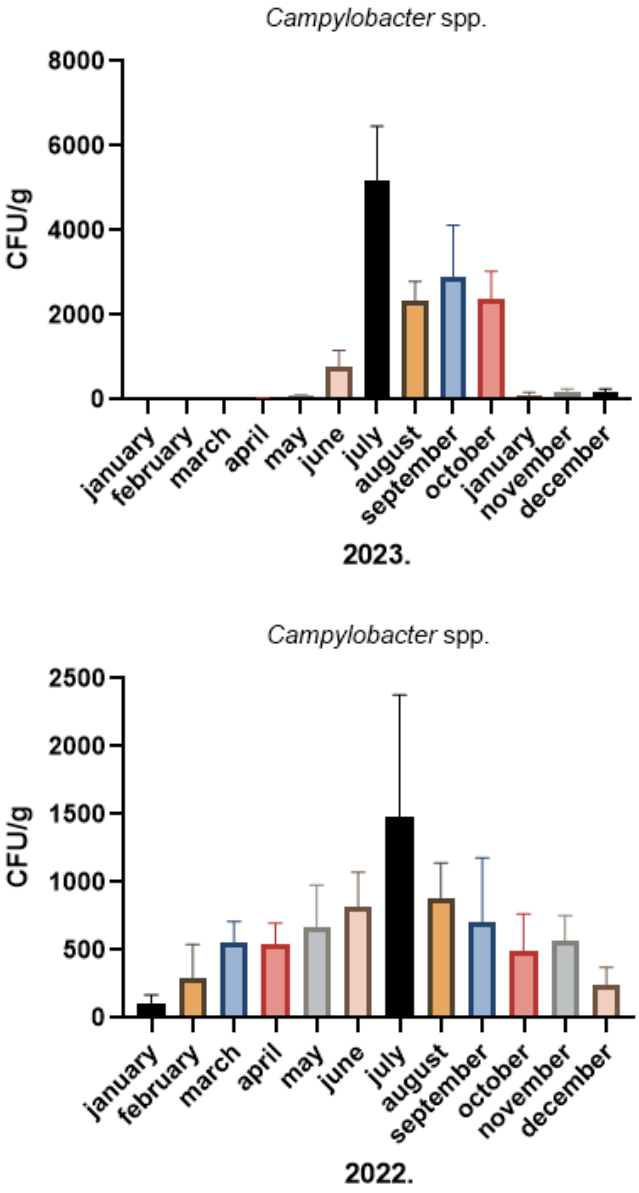

Figure 2 Occurrence of *Campylobacter* in poultry samples in every month during 2022. and 2023.

Table 1 Descriptive statistical analysis of the number of *Campylobacter* spp. (CFU/g) on broiler chicken carcasses by sampling seasons.

Campylobacter spp. (CFU/g)							
Year	Season	X	SD	SG	Xmin	Xmax	CV (%)
2022	Spring	592	208.9	53.94	280.0	960.0	35.29%
	Summer	1058	604.8	156.2	420.0	3000	57.17%
	Autumn	589.3	320.6	82.78	160.0	1400	54.40%
	Winter	214.7	173.5	44.79	60.0	710.0	80.82%
2023	Spring	32.00	41.78	10.79	0.000	120.0	130.6%
	Summer	2760	2034	525.1	250.0	6800.0	73.69%
	Autumn	1822	1423	367.4	110.0	4700	78.10%
	Winter	62.67	99.46	25.68	0.000	260.0	158.7%

Legend: X – arithmetic mean; SD – Std. Deviation

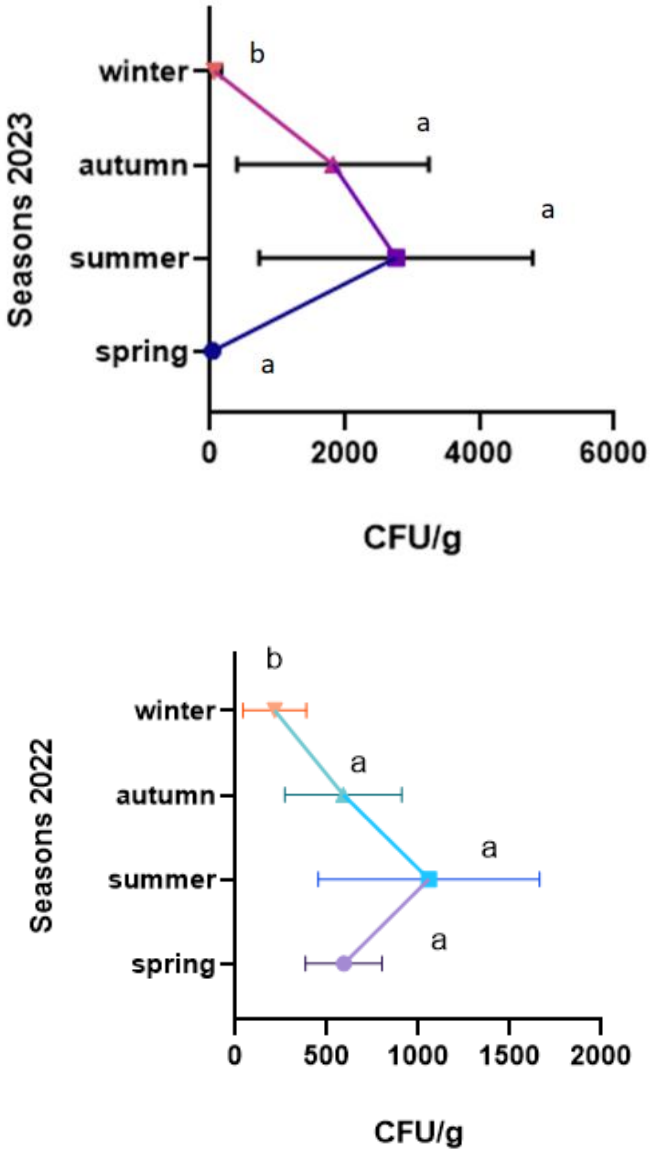

Figure 3 Effect of seasonal variations on the prevalence of *Campylobacter* spp. (CFU/g). Statistical significance is shown with the different letters: a - P<0.001

A significant correlation coefficient was found between *Campylobacter* counts (P<0.001) obtained in the winter seasons compared to counts obtained during other seasons in both sampling years (Fig. 1).

In this study, the correlation between the occurrence of these two bacterial species in the same samples of broiler chicken carcasses were examined. Based on the results of this research, it can be concluded that the hygiene of the production process in the facility for the production and processing of poultry meat was satisfactory in 135 samples and nosatisfactory (>1000 CFU/g) in all others samples, based on the limit values of the Commission Regulation (EC) No 2017/1495 and Rulebook on general and special hygiene conditions at any stage of production, processing and circulation (**"Official Gazzete of the RS", No. 72/2010 & 62/2018**). **Process hygiene in poultry processing plants is critical for ensuring food safety and minimizing microbiological contamination. By determining the level of presence of *Salmonella* and *Campylobacter* species at the slaughter line, the effectiveness of all previously implemented hygiene measures on the farm, during transport, and throughout the slaughter and processing process is confirmed.** According to previous reports in EU (European Union) and Serbia, *Salmonella* and *Campylobacter* are the most common pathogens that seriously affect public health. Since 2005, campylobacteriosis has been the most commonly reported zoonosis, and this trend continued in 2022. *Campylobacter* is also one of the major causes of foodborne bacterial gastroenteritis also in Serbia.. In Serbia, the surveillance of enteritis caused by *C. coli/jejuni* has been carried out since 1997, and monitoring of these bacteria in poultry carcasses has been mandatory since January 1, 2019. According to the report by the Institute of Public Health of Serbia, "Dr Milan Jovanovic Batut," on infectious diseases in 2022, the incidence of human campylobacteriosis was 4.04/100.000, or 276 reported cases. Average annual incidence, from 2005 to 2017, of enteritis caused by *Campylobacter* in Serbia was 5.30 per 100.000 population. It shows a decreasing trend in our country. Campylobacter prevalence in poultry, especially in broiler could be as high as 100% (**Asmai *et al*., 2020**). From the total number of examined boiler chicken carcass samples in the present study (360), 306 samples were identified as *Campylobacter* spp., which represents a occurrence of 85%. These results highlight how often contaminated broiler meat reaches consumers and show the higher frequency than the average for the EU. Of the 429 broiler chickens samples from the EU, 141 (32.9%) had Campylobacter species. The fact is that the frequency of these bacteria in the EU varies significantly in different member countries - 3 (1.8%) in Estonia, 49 (36.6%) in Latvia, and 89 (66.9%) in Lithuania) (**EFSA, 2022**). The findings of this study on the occurrence of *Campylobacter* in neck skin samples are in agreement with reports from Algeria (80%) (**Messad *et al.*, 2014**), and it is higher than the prevalence rates of 24.4% and 27.4% reported in Switzerland and Sri Lanka, respectively (**Frediani-Wolf *et al.*, 2023; Kottawatta *et al.*, 2017**). Concerning positive samples in EU, 19.4% exceeded the limit of 1000 CFU/g. This is in line with the results of this study, where 27.45% of all positive samples exceeded the limit set by EU regulations.

The World Health Organization (WHO) and the Food and Agriculture Organization (FAO) declared *Salmonella* as the most common and important zoonosis since 1950 (**Mouttotou *et al*., 2017**). In Serbia, according to the report by the Institute of Public Health of Serbia "Dr Milan Jovanovic Batut" on infectious diseases in 2022, the incidence of human salmonellosis was 7.51/100.000, or 513 reported cases. This number is still on a downward trend. In this investigation, the percentage of broiler chicken carcasses contaminated with *Salmonella* decreased during 2023 by more than 50%. From the total number of examined broiler chicken carcass samples in this study (360), *Salmonella* spp. was detected on 84 (23.33%). A greater number of positive samples were obtained by researchers from Portugal (**Antunes *et al*., 2003**), who examined the presence of *Salmonella* on 60 broiler chicken carcasses, and the presence of these bacterium was found in 60% of the samples. Similar analyses was conducted in Spain, on a larger number of samples (336 broiler carcasses), of which 17.9% were positive for Salmonella (**Capita *et al.*, 2007**). According to EFSA report, the most often isolated serovars in poultry are: *S. Enteritidis, S. Typhimurium,* and *S. Infantis*. They are change over time and depend from country and region (**EFSA, 2019**). This study shows the presence of three different *Salmonella* serovars in broiler chickens and the most common were *S. Enteritidis* (68%) and *S. Infantis* (18%), which is in agreement with te previous reports in Serbia. The most often isolate serovars in the parts of the Autonomous Province of Vojvodina were *S. Infantis* (50.3%) and *S. Enteritidis* (48.2%) (**Pajić *et al*., 2015**). **Spalevic *et al.* (2023)** reported that serovars *S. Enteritidis* and *S. Infantis* were the most often isolated in Belgrade region, but in 2018-19 the occurrence of *S. Mbandaka* and *S. Senftenberg* was also detected.

In this investigation, no correlation was observed between the *Salmonella* positive samples and amount of *Campylobacter* isolated from the same samples, indicating that both bacteria exist independently of one another. Similar results were obtained by other authors (**Cason *et al*. 1997, Wilson *et al.*, 2002**), who reported that the occurrence of these two bacteria is independent, meaning that co-contamination

does not occur. This means that implementing certain control measures will reduce *Salmonella* contamination but will not be effective against *Campylobacter*.

Several factors can affect the occurrence of these bacteria in broiler meat. The most common factors are environmental, as well as the season in which the samples are taken (**Ichiro *et al.*, 2017**). In this study, this phenomenon was investigated and the relationship between the season and the prevalence of these pathogens was determined, so samples were collected during each month, covering all four seasons. Sampling season exhibited a significant impact on the occurrence of *Campylobacter* and *Salmonella*, with peak recorded in summer ($p<0.001$). Similar results were obtained from other researchers (**Balli *et al.*, 2020**). The reasons for the seasonal variation associated with these pathogens are undefined. However, possible associations between the higher presence of pathogens and the temperature, and/or the extensive availability of additional reservoirs (domestic and wild animals) for the disease could explain the high occurrence in summer (**Patrick *et al.*, 2014; Ellis-Iversen *et al.*, 2009**). In countries with temperate climates, like Serbia, commonly observed seasonal peaks of campylobacteriosis in humans occur mostly in June and July. In line with that, higher isolation rates of *Campylobacter* from broiler chickens in June, July, and August compared to the winter months have been observed (**Ozbey *et al.*, 2014; Williams *et al.*, 2015**). In contrast, a study conducted in Norway showed results different from the present study, where the peak of Campylobacter occurs at the end of summer and the beginning of autumn (**Kapperud *et al.*, 1993**). The exact explanation for these differences still does not exist, but it is believed that they can occur due to different climatic conditions that prevail in different parts of the world. The occurencce of *Campylobacter* infection also depends on the quantity of broiler chicken meat consumed and the method of preparation (**Skarp *et al.*, 2016**). The current socioeconomic situation in Serbia can also be one of the reasons for the pronounced seasonal occurrence and presence of these bacteria in poultry meat. According to field data, a good part of small farms that have poorer biosecurity measures (higher risk of pathogen transmission) do not fatten chickens in winter due to expensive heating but only fatten chickens in the warmer months.

CONCLUSIONS

Based on the results obtained in this study, we can conclude that the presence of *Campylobacter* spp. and *Salmonella* spp. in broiler meat presents a significant public health issue in Serbia. Doubling poultry meat production in recent years has clearly affected the global burden of campylobacteriosis and salmonellosis and will put further pressure on the poultry industry and public health. The implementation of specific control procedures, monitoring, and preventive approaches such as HACCP from the farm through to the consumer is recommended to reduce the incidence of these pathogens and the risk to consumers. Despite the implementation of all necessary preventive measures, the persistence of these pathogens in poultry meat remains a challenge and still requires continuous monitoring and improvement of food safety practices. More research is necessery to determine prevalence of Salmonella and Camylobacter in Serbia and to develop novel intervention strategies to decrease broiler carcass contamination.

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