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Detection of bacteria of the genus *Salmonella* in meat from northwest Croatia

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Abstract

Salmonellosis is one of the most common food-borne zoonoses, and the most common source of infection for humans is meat and meat products. In 2023, 1125 samples of portioned meat (chicken, pork, beef) were tested in butcher shops in five counties of northern Croatia. The samples were tested for the presence of bacteria of the genus *Salmonella*, which were isolated in 88 (7.82%) samples. *Salmonella* was isolated from 69 of 604 (11.4%) chicken meat samples, from 10 of 319 (3.1%) pork samples and from 9 of 202 (4.5%) beef samples. Serological typing of chicken meat samples identified *S*. Infantis in 60 (87%) isolates, *S*. Mbandaka in six (8.7%), and *S*. Enteritidis in three (4.3%) samples. In pork, *S*. Derby was identified in two (20%) isolates, *S*. Typhimurium, *S*. Typhimurium (monophasic), *S*. London and *S*. Rissen in one isolate each (10%). In beef, *S*. Bovismorbificans was detected in five (55.6%) and *S*. Muenster in one (11.1%) isolate. Salmonellosis can be an economic problem due to the damage it causes in intensive production, but also a significant public health challenge as it spreads through the environment via consumption and the handling of contaminated meat in slaughterhouses, food processing and retail facilities. The implementation of appropriate hygiene measures and good hygiene practices from farms and slaughterhouses to butchers could help to reduce the contamination of meat with bacteria of the genus *Salmonella*.

Key words: *salmonellosis; meat; food safety; Salmonella; serovar*

Introduction

Among food-borne zoonoses, salmonellosis is one of the most frequently reported zoonoses in the European Union. It is caused by bacteria of the genus *Salmonella*, which have been shown to occur in all species of mammals, birds, fish, reptiles, amphibians and invertebrates. Humans most commonly become infected through contaminated food, often due to poor hygiene practices, direct and indirect contact with pets such as dogs, cats and reptiles and their environment, and infected animals (Wibisono et al., 2020). The consumption of food and water con-

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taminated with *Salmonella* can lead to illness. The main sources of infection for humans are contaminated meat and meat products, eggs, raw milk and products made from raw milk, fish and seafood, chocolate and other sweets, and fruit and vegetables, especially leafy vegetables (Andino and Hanning, 2015; Fischerström et al., 2023). Contamination of meat can occur in slaughterhouses after slaughter during evisceration, through intestinal contents, in meat processing plants, and in butcheries through cross-contamination (Manfreda et al., 2014). The pathogen can survive for months under unfavorable conditions, as it is able to form biofilms consisting of several bacterial species that protect it from unfavorable environmental influences (Chen et al., 2024). Different *Salmonella* species are widely distributed in the environment and in the physiological microflora of numerous organisms. Within two species, there are many subspecies and serovars. A total of 46 O and 114 H antigens have been identified, which are labeled with numbers and letters and whose combinations give the antigenic formula of the different serovars (Brenner et al., 2000; Issenhuth-Jeanjean et al., 2014). They can be categorized into several groups based on their ability to infect and their pathogenicity to specific hosts. Those that cause infection in a wide range of hosts differ genetically through changes in virulence plasmids and chromosomal rearrangements, leading to various clinical manifestations (Connor and Schwartz, 2005; Silva et al., 2014; Soliani et al., 2023). They are highly adaptable and increasing antimicrobial resistance has been observed, causing enormous economic damage and representing one of the most common public health threats related to food production (Antunes et al., 2016). Figueiredo et al. (2015) described antibiotic resistance in *Salmonella*, with over 70% of the samples analysed resistant to at least one antibiotic. Monophasic *S*. Typhimurium and *S*. Infantis show exceptionally high resistance to several types of drugs. Virulence plasmids play a crucial role as they contain genes associated with antibiotic resistance and virulence factors, such as spvB (ADP-ribosylating toxin) and spvC (which inhibits pyroptosis and inflammation). It is important to note that serotypes with higher prevalence are more likely to develop resistance to commonly prescribed antibiotics. *Salmonella* is a highly virulent pathogen and the presence of bacteria at 10 CFU/ml poses a risk (Lamichhane et al., 2024). *Salmonella* strains, which cause disease in a wide range of hosts, are genetically more diverse and lead to different clinical manifestations (Silva et al., 2014). In 2022, 65,208 cases of human salmonellosis were confirmed in the European Union Member States, corresponds to a reporting rate of 15.3% per 100,000 inhabitants. The five most frequently reported serovars causing salmonellosis in humans are *S*. Enteritidis (67.3%), *S*. Typhimurium (13.1%), monophasic *S*. Typhimurium (4.3%), *S*. Infantis (2.3%) and *S*. Derby (0.89%). In Croatia, 1,047 (21.7%) cases of human salmonellosis were confirmed in 2022 (EFSA, 2023).

To prevent the zoonotic transmission of *Salmonella*, national salmonella control programmes are implemented in European countries. In Croatia, there are annual monitoring programs for salmonella in poultry flocks (laying hens, chicks and turkeys), and carcasses of pigs, cattle and sheep are tested in slaughterhouses, as are neck skins of chicken carcasses, meat products, meat preparations and minced meat. In addition, food producers have defined criteria for salmonella in their HACCP plans.

Despite all these efforts, this disease persists. The aim of this study was to determine the prevalence of contamination of portioned meat and small pieces of meat with *Salmonella* bacteria and the frequency of certain serovars in five counties in northern Croatia.

Methods

In 2023, 1125 samples of chicken, pork and beef categorised as portioned meat and small pieces of meat were collected in butcheries in Međimurje, Varaždin, Koprivnica-Križevci, Bjelovar-Bilogora and Zagreb Counties, and tested in the laboratory for the presence of *Salmonella* bacteria. The meat was sampled at the butcher's shops before the expiry date, and samples were transported in portable coolers under controlled conditions to maintain the cold chain to the Laboratory of Food and Feed Microbiology of the Veterinary Institute in Križevci, where testing began immediately.

Isolation of Salmonella Bacteria

For the isolation of *Salmonella* bacteria, the method according to the standard HRN EN ISO 6579-1:2017; HRN EN ISO 6579-1:2017/A1:2020 was used. The test sample consisted of 25 g meat diluted with 225 mL buffered peptone water (Biokar, France). MkTTn (Biokar, France) and RVS (Oxoid, UK) broths were used for selective enrichment and two selective media were used for Salmonella growth: XLD (Biokar, France) and RAMBACH agar (Merck, Germany). After incubation for 24 hours at 37°C, *Salmonella* colonies on XLD agar show a black center and a slightly transparent reddish zone due to the color change of the indicator, while they appear red on Rambach agar. Colonies grown on XLD and Rambach agar were subcultured on neutral nutrient agar (Merck, Germany).

Identification of Bacteria

Confirmatory tests for the identification of *Salmonella* included TSI agar (Merck, Germany), Gram staining, oxidase test (Merck, Germany) and biochemical identification using the automated Vitek2 Compact system (bioMerieux, France) with the Vitek2 ID GN identification card. A pure culture grown on neutral agar was transferred with a sterile swab into a sterile solution (0.45-0.50% NaCl, pH 4.5-7.0) to obtain a bacterial suspension with an optical density of 0.50 to 0.63 according to

Table 1. Overview of biochemical tests in the VITEK2 system in the case of confirmed presence of *Salmonella* spp.

Biochemical Details																	
2	APPA	–	3	ADO	–	4	PyrA	–	5	1ARL	–	7	dCEL	–	9	BGAL	–
10	H2S	+	11	BNAG	–	12	AGLTp	–	13	dGLU	+	14	GGT	–	15	OFF	+
17	BGLU	–	18	dMAL	+	19	dMAN	+	20	dMNE	+	21	BXYL	–	22	BAlap	–
23	ProA	–	26	LIP	–	27	PLE	–	29	TyrA	–	31	URE	–	32	dSOR	+
33	SAC	–	34	dTAG	–	35	dTRE	+	36	C1T	+	37	MNT	–	39	5KG	–
40	lLATk	+	41	AGLU	–	42	SUCT	+	43	NAGA	–	44	AGAL	+	45	PHOS	+
46	GlyA	–	47	ODC	+	48	LDC	+	53	lHISa	–	56	CMT	+	57	BGUR	–
58	O129R	+	59	GGAA	–	61	lMLTa	–	62	ELLM	–	64	lLATa	–			

McFarland. Colorimetric cards for the identification of Gram-negative bacteria (bioMerieux, France) containing the tests shown in Table 1 were used for the identification of *Salmonella*.

The results of the biochemical tests with the VITEK2 system were reported at the end of the measurements with the percentage of certainty of identification. Polyvalent and monovalent sera (SSI Diagnostica, Denmark) were used for serological confirmation and identification. The presence of *Salmonella* O-, H- and Vi-antigens was detected by agglutination of pure colonies using the rapid agglutination technique on a slide with the corresponding serums after elimination of auto-agglutinating strains. The nomenclature of serovars and the serological typing scheme were performed according to the White-Kauffmann-Le Minor scheme (Grimont and Weill, 2007). We used the software program Stata 13.1 (Stata Corp., USA) to analyse the numerical data collected in the study. The association between the frequency of *Salmonella* isolation and the type of meat was assessed using the chi-square test. The results of the statistical analysis were expressed as a probability (*P*-value), and values less than 0.05 were considered statistically significant.

Results

During the study period, 1125 meat samples were processed: 604 chicken, 319 pork and 202 beef samples. *Salmonella* bacteria were isolated from 88 (7.8%) of the total processed samples (Table 2). The highest number was isolated from chicken meat, with 69 (11.4%) samples, followed by 10 (3.1%) samples of pork and nine (4.5%) samples of beef. Serological typing identified *S.* Infantis in 60 (87%), *S.* Mbandaka in six (8.7%) and *S.* Enteritidis in three (4.3%) isolated samples of chicken meat. In pork, *S.* Derby was identified in two (20%) isolates, while *S.* Typhimurium, monophasic variant (1,4 [5],12:i:-), *S.* London and *S.* Rissen were identified in one (10%) isolate each. In beef, *S.* Bovismorbificans was identified in five (55.6%) isolates and *S.* Muenster

Table 2. Number of Processed Samples and Samples with *Salmonella*

Type of meat	Number of Processed Samples	Number of Positive Samples	(%)	Identified Salmonella Serovars	Number	(%)
Portioned Chicken	604	69	11.4	S. Infantis	60	87
				S. Mbandaka	6	8.7
				S. Enteritidis	3	4.3
Portioned Pork	319	10	3.1	S. Derby	2	20
				S. London	1	10
				S. Typhimurium	1	10
				S. Typhimurium (monophasic)	1	10
				S. Rissen	1	10
				S. spp	4	40
Portioned Beef	202	9	4.5	S. Bovismorbificians	5	55.6
				S. Muenster	1	11.1
				S. spp	3	33.3
Total	**1125**	**88**	**7.8**			

(11.1%) in one isolate. Four isolates from pork and three isolates from beef were biochemically confirmed to the genus level but not serologically typed to the species level. The correlation between the frequency of *Salmonella* isolation and the type of meat was determined using the chi-square test. The frequency of isolation of *Salmonella* bacteria was highest in portioned chicken meat (11.42%) and lowest in portioned pork (3.13%). The observed differences in the frequency of *Salmonella* isolation from portioned meat and small pieces of meat from different animal species were statistically significant (*P*<0.001).

Discussion

In this study, 1125 meat samples were processed, and *Salmonella* bacteria were isolated from 88 (7.82%) of samples. In chicken meat samples, *Salmonella* was found in 11.4% of the samples, with the serovar *S*. Infantis confirmed in 87% of isolated strains, followed by *S*. Mbandaka in 8.7% and *S*. Enteritidis in 4.3% of the isolates. The results of this study indicate an increase in the prevalence of *Salmonella* in chicken meat compared to previous periods. Listeš et al. (2013) detected *Salmonella* spp. in 8.3% of poultry meat samples in Dalmatia from 2007 to 2009, 4.5% in meat preparations, 3.4% in slaughter meat, 2.2% in processed meat and 0.1% in portioned meat and meat preparations. The most frequently occurring serovars were *S*. Typhimurium, *S*. Enteritidis, *S*. Montevideo, *S*. Tennessee and others, with 19 different serovars typed. Jaki Tkalec et al. (2021) concluded that the prevalence of *Salmonella* in chicken meat from farms in northern Croatia was 5.5% from 2016 to 2020. In 2016, it was found in 6% of the samples tested, in 2017 in 4.7%, in 2018 in 6.1%, in 2019 in 6.6% and in 2020 in 5.4% of the samples. In serological typing, *S*. Infantis was identified in 63.2% of the isolated strains, making it the most frequently confirmed serovar in all years. *S*. Typhimurium was isolated in 5.9% of the strains, *S*. Enteritidis in 2.2% and *S*. Mbandaka in 0.7% of isolated strains, together with 12 other serovars. Hengl et al. (2016) found that the prevalence in Croatia was higher at 10.5%, based on a study of 474 chicken meat samples and products collected during official hygiene inspections. The most common serovar was *S*. Infantis, with other serovars such as *S*. Thompson, *S*. Enteritidis, *S*. Typhimurium and *S*. Agona identified. In Eastern Croatia, the presence of *S*. Infantis was detected in 14.8% of 837 samples of portioned chicken meat (Perković et al., 2021), which represents a slightly higher prevalence compared to our results. Pate et al. (2019) showed a prevalence of 28.4% for *Salmonella* in fresh broiler meat and 26.7% in meat preparations from broilers in Slovenia, with *S*. Infantis detected in 4.5% of samples. According to the EFSA Annual Report 2022, fresh chicken meat and chicken products continue to be the category in which *Salmonella* is most frequently detected, and *S*. Infantis is the most prevalent serovar. It is believed that the vaccination of poultry with the serovars *S*. Typhimurium and *S*. Enteritidis has led to an increase in *S*. Infantis, which has occupied an ecological niche and now dominates poultry farms in the EU (Andino and Hanning, 2015). Despite the implementation of all hygiene measures during the plant closure period, this pathogen remains present on certain farms (Pate et al., 2019).

Pork is one of the most commonly consumed meats in the European Union, and *Salmonella* is one of the major biohazards in pork, especially the serovar Typhimurium, including its monophasic variant worldwide. *Salmonella* is commonly found in the tonsils, intestines, mesenteric lymph nodes and parotid glands and spreads to the carcasses of pigs during slaughter processing, as well as to the entire production line, equipment, knives and workers (Salines et al., 2023). During the study, *Salmonella* was detected in 3.1% of pork samples, and serotyping identified *S*. Derby in 20% of isolates, *S*. Typhimurium, *S*. Typhimurium (monophasic), *S*. London and *S*. Rissen in 10% of isolated strains. The results of a study carried out in seven abattoirs in southern Germany on various pork samples (e.g., carcasses, livers, kidneys, tongues, hearts, lungs) showed a *Salmonella* prevalence of 1.1% (Meyer et al., 2010). The highest percentage of samples testing positive for *Salmonella* by isolation was found in pork tongues (5.0%), suggesting that tongues can be a source of cross-contamination of meat when consumed undercooked. In the same study, *Salmonella* was isolated from 17 of 1569 pig carcasses (1.1%). A high prevalence was reported in Belgium (37%), where it was concluded that the high contamination of carcasses after slaughter was due to the delivery of pigs positive for *Salmonella*, accidental spillage of caecal contents, mainly feces, during evisceration and cross-contamination from the slaughterhouse environment (Botteldoorn, 2003). Deane et al. (2022) isolated *Salmonella* from swab samples of pig carcasses in 11.5% of samples and in lymph nodes in 31.7%. *S*. Typhimurium, including the monophasic variant, was the most frequently isolated serovar, found in 56% of isolates from swabs of carcasses and in 73% from lymph nodes. *S*. London and *S*. Derby were the next most frequently isolated serotypes. Contamination of carcasses with *Salmonella* is an important parameter to measure the effectiveness of hygiene criteria in slaughterhouses. These results confirm the persistently high levels of *Salmonella* in fattening pigs, and the high prevalence of *Salmonella* in lymph nodes indicates that *Salmonella* remains a significant problem in slaughter and hygiene compliance in slaughterhouses. In 2011, EFSA conducted a study in the EU Member States, Iceland and Norway, in which a total of 52,868 units of fresh pork were tested, with 0.7% testing positive for *Salmonella* (EFSA, 2013). A study on the prevalence and diversity of *S.* Enterica serovars in animal-derived foods (beef, pork, poultry and seafood) on five continents (Africa, North and Latin America, Asia, Europe and Oceania) shows that the serovar Typhimurium is ubiquitous on all continents, with Africa and Europe leading the way (Ferrari et al., 2019).

In our analysis of beef samples, *Salmonella* was found in 4.5% of the samples tested. Among the serovars isolated, *S.* Bovismorbificans was confirmed in 55.6% and *S.* Muenster in 11.1% of isolates. In the USA, most *Salmonella* outbreaks were caused by ground beef prepared at home, and studies show that one-third of Americans do not use safe food handling practices to avoid cross-contamination in the kitchen (Laufer et al., 2015). Bosilevac et al. (2008) found a prevalence of 4.2% in ground beef from seven regions of the USA, which is very similar to our results. The most frequently isolated serotypes were *S*. Anatum, *S*. Mbandaka, *S*. Montevideo and *S*. Muenster, which accounted for 50% of the isolates. Meyer et al. (2010) found *Salmonella* in only one sample (0.1%) in a study of 1366 samples of beef (swabs, carcasses, liver, kidney, tongue, heart, lung). The most common serovars in cattle are *S*. Typhimurium and *S*. Dublin, in pigs they are *S*. Typhimurium (monophasic), *S*. Derby and *S*. Typhimurium. In broilers, the most common serovars are *S*. Infantis, *S*. Enteritidis and *S*. Mbandaka. In the EU, *S*. Enteritidis, *S*. Typhimurium and monophasic *S*. Typhimurium are the most common causes of salmonellosis in humans. In fresh meat, the highest number of positive samples was found in broiler meat, which is understandable considering that national *Salmonella* control programmes are implemented in the EU for the poultry population to reduce *Salmonella*, while there is no coordinated approach between EU Member States for other animal species. Comparing the number of reported cases of salmonellosis caused by the consumption of food contaminated with salmonella in 2022, a slight increase was observed in Croatia and the EU compared to 2021 and 2020. The proportion of monophasic *S*. Typhimurium has slightly increased, while the proportion of *S*. Enteritidis has decreased compared to previous years, when this strain was in second place in Europe and third place in the USA. This could be due to the continuation of everyday activities (social events, visits to the doctor, travel) after the restrictions due to the COVID-19 pandemic (EFSA, 2023).

Conclusions

This research, which revealed the presence of *Salmonella* bacteria at a level of 11.4% in chicken meat, 3.1% in pork and 4.5% in beef from butcher shops in northwest Croatia, indicates an increase in the incidence of *Salmonella*. *Salmonella* spp. has always been a global public health concern as it can be transmitted to humans through consumption and spread in the environment when handling contaminated meat in slaughterhouses, processing plants and households. The emergence of antibiotic resistance in *Salmonella* poses a major challenge in the treatment of *Salmonella* infections, as it often leads to treatment failures and associated costs. Salmonellosis is also a significant economic problem as it damages livestock production. It is important to identify the factors that contribute to the spread of this pathogen in meat production in order to reduce the risk of salmonellosis cases in humans. Therefore, monitoring for pathogens in fresh meat and products at the end of the production chain is crucial. The implementation of appropriate hygiene measures and good hygiene practices from farms and slaughterhouses to butchers could help to reduce *Salmonella* contamination.

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Nalaz bakterija iz roda *Salmonella* u mesu s područja sjeverozapadne Hrvatske

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Salmoneloza je jedna od najučestalijih zoonoza koja se prenosi hranom, a najčešći izvor zaraze za ljude je meso i proizvodi od mesa. Tijekom 2023. godine uzorkovano je 1125 uzoraka porcioniranog mesa (pileće, svinjsko, juneće) u mesnicama na području pet županija sjeverne Hrvatske. Uzorci su pretraženi na prisustvo bakterija iz roda *Salmonella* koje su izdvojene u 88 (7,82 %) uzoraka. Od 604 uzoraka pilećeg mesa salmonele su izdvojene u 69 (11,4 %) uzoraka, od 319 uzoraka svinjskog mesa u 10 (3,1 %) uzoraka i od 202 uzorka junećeg mesa u 9 (4,5 %) uzoraka. Serološkom tipizacijom *S*. Infantis je identificirana u 60 (87 %) izolata iz uzoraka pilećeg mesa, *S*. Mbandaka u 6 (8,7 %), a *S*. Enteritidis u 3 (4,3 %). Iz svinjskog mesa *S*. Derby identificirana je u 2 (20%) izolata, *S*. Typhimurim, *S*. Typhimurium (monofazna), *S*. London i *S*. Rissen u po jednom (10 %) izdvojenom izolatu. Iz junećeg mesa identificirane su *S*. Bovismorbificans u 5 (55,6 %) i *S*. Muenster u jednom (11,1 %) izolatu. Salmoneloza može predstavljati gospodarski problem zbog šteta koje nanosi u intenzivnoj proizvodnji, ali i znatan javnozdravstveni izazov jer se konzumacijom i manipulacijom kontaminiranog mesa širi na okoliš u klaonicama, objektima prerade i prodaje hrane. Provedbom odgovarajućih higijenskih mjera i dobre higijenske prakse od farmi i klaonica do mesnica moglo bi se doprinijeti manjoj kontaminaciji mesa bakterijama iz roda *Salmonella*.

Ključne riječi: *salmoneloza, meso, sigurnost hrane, Salmonella, serovar*