Occurrence of *Salmonella* spp. in flocks of laying hens in different housing systems in eastern Croatia from 2020 to 2023

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Abstract

Human *Salmonella* infections are often associated with the consumption of infected eggs and egg products. Therefore, knowing the prevalence of *Salmonella* spp. in flocks of laying hens and the factors that influence the occurrence of *Salmonella* on farms is of the utmost importance. Since 2012, housing laying hens in conventional cages has been prohibited in the European Union, and hens may only be kept in enriched cages or alternative housing systems. The prohibition of conventional cages has improved the welfare of laying hens, but has raised issues concerning the detrimental consequences for the spread and occurrence of infectious diseases in hen flocks. The aim of this study was to analyse the occurrence of *Salmonella* spp. in laying hen flocks in eastern Croatia in the period from 2020 to 2023, and to determine whether housing systems have an impact on occurrence. During this period, a total of 1534 samples of laying hen faeces from 191 flocks (106 flocks housed in enriched cages and 85 flocks in housing systems with closed outdoor runs) were tested at the Diagnostics Laboratory of the Croatian Veterinary Institute, Veterinary Department Vinkovci, as part of implementation of the National Salmonellosis Control Programme and official sampling in the Republic of Croatia for the presence of *Salmonella* spp. Isolation, identification and serotyping of *Salmonella* was conducted in accordance with the standard HRN EN ISO 6579-1:2017/A1:2020 method. *Salmonella* spp. was confirmed in the faeces of seven laying hen flocks (6.60%) housed in enriched cages, and 11 flocks (12.94%) of laying hens in housing systems with outdoor runs. The research results showed that there was no significant difference in the total occurrence of *Salmonella* between flocks of laying hens housed in housing systems with outdoor runs and those in enriched cages. The highest percentage of positive flocks was found in 2020 in 11.11% of flocks of laying hens housed in enriched cages and 18.18% of flocks of laying hens kept in housing systems with outdoor runs.

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When observed by county over the four-year study period, the percentage of positive flocks was significantly higher ($P<0.05$) in Brod-Posavina County in comparison with Osijek-Baranja County and Vukovar-Srijem County. The research results suggest that there is small possibility that transitioning from enriched cage systems to alternative housing systems, with strict biosecurity measure implementation, would increase *Salmonella* infections in laying hen flocks.

Key words: *laying hens*; *Salmonella spp.*; *housing system*; *enriched cages*; *alternative housing systems*

Introduction

Pursuant to Council Directive 1999/74/EC, the housing of laying hens in conventional cages has been prohibited in the European Union (EU) since 2012. With the aim of improving hen welfare, the Directive prescribed that laying hens may be housed in either enriched cages or in alternative (non-cage) housing systems. Alternative systems include floor housing (single level), aviaries (multi-levels) and outdoor systems (Elson, 2011; Potori et al., 2023). As animal welfare is an increasingly important issue among egg consumers, the EU is increasingly supporting abandoning cage systems. The campaign entitled "End the Cage Age" was launched in 2018 and resulted in the collection of 1.4 million signatures in a Europe-wide petition to end the use of cage systems. On 30 June 2021, the European Commission announced a political initiative to gradually ban cages in the EU (EC, 2021), which would have a significant impact on the egg industry. Enriched cages for laying hens are banned in Luxembourg and Austria, with Germany (as of 2025 and in exceptional cases in 2028) and Slovakia (signed memorandum between the government and industry as of 2030) set to join. In September 2020, the Czech parliament voted for a ban on cage housing of laying hens as of 2027. In the EU, more than 50% of laying hens are now kept in alternative systems (Majewski et al., 2024). The effects of different alternative systems have been researched in detail and assessed in the context of the welfare of laying hens and economic feasibility, though the question arises as to the effects on the occurrence and spread of contagious diseases, and ultimately on public health (Holt et al., 2011). These issues are not surprising since one of the main advantages of conventional cages is that the hens are separated from their faeces, thereby reducing the risk of disease transmission to a minimum (Duncan, 2001). Accordingly, the bacteriological safety of eggs and egg products from laying hens held in alternative systems has become an important factor that needs to be monitored. In that sense, bacteria from the genus *Salmonella* are among the most common causative agents of human diseases around the world. In the EU, *S*. Enteritidis (54.6%), *S*. Typhimurium (12.1%) and monophase *S*. Typhimurium (10.4%) are the three most common serotypes causing salmonellosis in humans (EFSA and ECDC, 2023). Sources of infection of *S*. Enteritidis in humans are primarily eggs and egg products (when consumed raw or insufficiently cooked), while infections of *S*. Typhimurium typically originate from pig, cattle and poultry meat, and due to environmental contamination caused by pets or infected birds (Martelli and Davies, 2012). Two independently conducted studies in Sweden and Switzerland showed an increase in the number of bacterial diseases in laying hens following the ban of conventional cag-

es (Fossum et al., 2009; Kaufmann-Bart and Hoop, 2009). Previous research has shown that mortality, contamination of egg shells, and infections with *Salmonella* spp. is higher in alternative than in conventional cage housing (Holt et al., 2011; Svobodová et al., 2014). In alternative systems, there is a higher percentage of dirty or cracked eggs, due to the possibility of laying eggs outside the nest on the floor (Englmaierová et al., 2014). The presence of a wide spectrum of *Salmonella* serotypes in poultry meat and egg production therefore poses a significant challenge for the poultry sector.

The aim of this study was to compare the occurrence of bacteria from the genus *Salmonella* in flocks of laying hens kept in different housing systems in eastern Croatia.

Materials and methods

Faeces samples were tested for the presence of bacteria of the genus *Salmonella* at the Diagnostics Laboratory of the Croatian Veterinary Institute, Veterinary Department Vinkovci, as part of the National Salmonellosis Control Programme and official sampling protocols in laying hen flocks in the Republic of Croatia. During a four-year period (2020–2023), a total of 1534 samples of laying hen faeces were tested from 191 flocks (106 flocks housed in enriched cages and 85 flocks housed in housing systems with closed outdoor runs) in three counties in eastern Croatia: Brod-Posavina County, Osijek-Baranja County and Vukovar-Srijem County. In facilities with enriched cages, flock size varied from 500 to 54,400 laying hens, mostly in Brod-Posavina County and Osijek-Baranja County, while in outdoor run housing systems, flock size varied from 350 to 3000 laying hens (mostly in Vukovar-Srijem County). Flocks consisted of hens from 22 to 95 weeks of age, vaccinated with commercially available *Salmonella* vaccines. At all farms, strict biosecurity measures were implemented, with abidance of the principle "all in-all out", and thorough cleaning and disinfection of structures between flocks. Samples were collected and delivered to the laboratory by authorised veterinary organisations.

For *Salmonella* spp. isolation, the international standard method was applied, i.e., horizontal method for proving the presence, and determination of number and serotyping *Salmonella* – part 1: Proving the presence of *Salmonella* spp. (HRN EN ISO 6579-1:2017/A1:2020). Testing was performed on a 25 g sample (shoe liner or faeces) to which 225 mL BPW (Buffered Peptone Water, Biolife, Italy) was added, then incubated at 34–38°C, for 18±2 hours. After incubation, faeces samples were inoculated onto semi-soft MSRV (Modified Semisolid Rappaport Vassiliadis, Biolife, Italy) medium such that the content of the inoculated peptone water was inoculated by puncturing the medium at three points with a quantity of 0.1 mL, then incubated at 41.5±1°C, for 24±3 hours. After incubation, bacteria from the MSRV medium in the microbial migration zone were looped onto selective nutrient media XLD (Xylose Lysine Deoxycholate Agar, Biolife, Italy) and CSA (Chromogenic *Salmonella* Agar Base, Biolife, Italy) and incubated at 34–38°C, for 24±3 hours. For biochemical confirmation of *Salmonella* spp., TSI (Triple Sugar Iron) agar, Urea Agar Base (Christensen), urea 40% solution, LI (Lysine Iron) agar (Biolife, Italy) and the automated Vitek2 Compact system (bioMerieux, France) were used. The results of biochemical testing on the Vitek2 system upon reading were expressed as the percentage of reliable identification. For serological

confirmation and identification, polyvalent and monovalent serums were used (SSI Diagnostica, Denmark). The presence of *Salmonella* O-, H- and Vi- antigens was proven using pure colony agglutination, rapid agglutination technique on appropriate sera, after elimination of auto-agglutinating strains. The nomenclature of serovars and serological typing were performed according to the White-Kauffmann-Le Minor method (Grimont & Weill, 2007). Isolated bacterial strains of *Salmonella* were confirmed at the National Reference Laboratory of the Croatian Veterinary Institute, Poultry Centre, Zagreb, Croatia.

Statistical analysis was performed using the program Statistica v. 14.1.0.8 (Cloud Software Group, Inc., 2023. Data Science Workbench, http://tibco.com) and the chi-square test. Statistical significance was set at $P<0.05$.

Results and Discussion

Of the total samples tested, 902 samples were from flocks of laying hens housed in enriched cages and 632 from flocks housed in housing systems with closed outdoor runs. During the four-year period, *Salmonella* spp. was confirmed in seven flocks (6.60%) housed in enriched cages and 11 flocks (12.94%) in housing systems with outdoor runs.

Table 1 shows that there were no significant differences in the percentage of positive flocks over the study years, except for 2021, and over the study period as a whole ($P>0.05$) between the two housing systems. Other authors, in particular Siemon et al. (2007) and Jones et al. (2012) found that there were no significant differences in the incidence of *Salmonella* infections or environmental contamination between cage and non-cage systems, while De Vylder et al. (2009) and Van

Table 1. Percentage of positive flocks of laying hens for *Salmonella* spp. in different housing systems in Brod-Posavina, Osijek-Baranja, and Vukovar-Srijem Counties in the period from 2020 to 2023.

Housing system	Year	No. tested samples/ flocks	No. positive samples/ flocks	% positive flocks of total tested flocks
Enriched cages	2020	232 / 27	6 / 3	11.11
	2021	228 / 30	0 / 0	0[a]
	2022	196 / 24	4 / 2	8.33
	2023	246 / 25	4 / 2	8.00
	Total	902 / 106	14 / 7	6.60
Housing systems with closed outdoor runs	2020	130 / 22	12 / 4	18.18
	2021	204 / 23	2 / 1	4.35[a]
	2022	144 / 19	6 / 3	15.79
	2023	154 / 21	6 / 3	14.29
	Total	632 / 85	26 / 11	12.94

[a] $P<0.05$

Hoorebeke et al. (2011) found there were no differences between conventional and enriched cage systems. In 2021, the percentage of positive flocks was significantly higher ($P<0.05$) in the housing systems with outdoor runs. In this year, *S.* Typhimurium was confirmed in only one flock of laying hens, i.e., in a flock in housing system with outdoor run. This can be associated with the influence of season on the occurrence of *Salmonella*. Since the infection of a flock positive for *S*. Typhimurium was confirmed in the fourth quarter, it is necessary to consider the influence of low external temperatures on the migration of rodents into poultry facilities and feed stores (Witkowska et al., 2018). It is known that rodent faeces can contaminate stored feed, and that this is the most common source of *Salmonella* infections in poultry (Heyndrickx et al., 2002). Lolić et al. (2022) conducted a ten-year study of *Salmonella* incidence in flocks of broilers and confirmed that *S*. Enteritidis was isolated in all seasons (quarters), while *S*. Typhimurium appeared only every few years, most often in the second and fourth quarters. Pieskus et al. (2008) confirmed the highest number of positive broiler flocks in spring, whereas laying hen flocks were mostly positive in winter, spring and autumn. Therefore, yearround biosecurity measures are necessary (Matković et al., 2019), with rat control as an essential biosecurity measure on farms (Backhans and Fellström, 2012).

The percentage of positive flocks for *Salmonella* spp. during the four-year period ranged from 1.89 to 14.29%, without any differences in individual years (Figure 1). Though a slight decline is visible from year to year, the percentage of positive in tested flocks remains high. In 2021, the percentage of positive flocks (1.89%) (Figure 1) approached the target of the National Salmonellosis Control

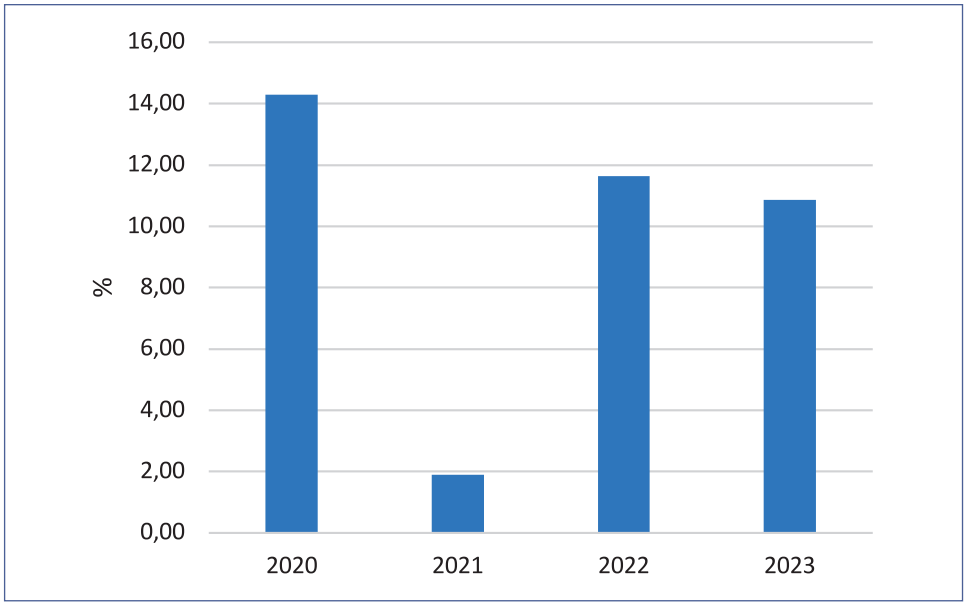

Figure 1. Percentage of positive flocks of laying hens in total flocks tested for *Salmonella* spp. in the period from 2020 to 2023.

Table 2. Percentage of positive flocks of laying hens in total flocks tested for *Salmonella* spp. in Brod-Posavina, Osijek-Baranja and Vukovar-Srijem Counties, in the period from 2020 to 2023

Year	County		
	Brod-Posavina	Osijek-Baranja	Vukovar-Srijem
	%		
2020	33.33[a]	0[b]	0[b]
2021	0[a]	0[a]	14.29[b]
2022	11.11[a]	10[a]	0[b]
2023	11.11[a]	10[a]	0[b]
Total	**13.51[a]**	**4.35[b]**	**4.35[b]**

[a,b] values in the same row marked with different letters differ significantly ($P<0.05$).

programme, emphasising the importance of reducing the incidence of *Salmonella* in adult flocks of laying hens from 2 to 1% at the annual level for the target serovars (*S*. Enteritidis and *S*. Typhimurium, including monophase variants) in abidance with the current testing schedule (EFSA, 2019).

Table 2 shows that the percentage of positive flocks in 2020 was significantly higher ($P<0.05$) in Brod-Posavina County than in Osijek-Baranja County and Vukovar-Srijem County. In 2021, the percentage of positive flocks was significantly higher ($P<0.05$) in Vukovar-Srijem County than in Brod-Posavina County and Osijek-Baranja County. As already explained, this can be attributed to the influence of season on the occurrence of *Salmonella*, i.e., influence of low external temperatures on the migrations of rodents into poultry facilities and feed stores. In 2022 and 2023, Brod-Posavina County and Osijek-Baranja County recorded significantly higher ($P<0.05$) percentages of positive flocks in comparison with Vukovar-Srijem County. The higher percentage of positive flocks in Brod-Posavina County and Osijek-Baranja County can be attributed to a higher number of flocks of laying hens housed in enriched cages, with a larger number of animals per facility, with older facilities used for egg production for a longer period of time, as discussed elsewhere (Mollenhorst et al., 2005; EFSA, 2007; Carrique-Mas et al., 2009; Huneau-Salaün et al., 2009; Gast et al., 2017). The older the facility, the more difficult it is to achieve a satisfactory standard of cleaning, due to wear and tear, age of equipment and the facility itself, particularly when considering that the level of contamination increases significantly during the production cycle (Wales et al., 2007). Furthermore, cage systems are more difficult to thoroughly clean and disinfect (Davies and Breslin, 2003; EFSA, 2007). In total, during the four study years, the percentage of positive flocks was significantly higher ($P<0.05$) in Brod-Posavina County than in Osijek-Baranja County and Vukovar-Srijem County. A study by Samper-Cativiela et al. (2023) from samples collected and tested as part of the National Salmonellosis Control Programme in a six-year period from 2015–2020 found that the incidence of *Salmonella* spp. was twice as high in flocks of laying hens kept in cage systems than in non-cage systems. Similar results

were reported by Methner et al. (2006), where the ratio of flocks positive for *Salmonella* was higher in conventional cages than in alternative housing systems. Numerous studies have assessed the effects of housing systems on *Salmonella* incidence in laying hens (EFSA, 2007; Namata et al., 2008; Snow et al., 2010; Van Hoorebeke et al., 2010b) and confirmed that laying hens housed in conventional cages had a significantly higher chance of infection with *Salmonella*. However, other authors report contrary results, including the present study, where there were no significant differences between housing systems for laying hens, or that there was a higher chance of contamination with *Salmonella* of laying hens and/or eggs housed in non-cage systems than in cage systems (De Vylder et al., 2011; Parisi et al., 2015; Jones et al., 2016; Van Hoorebeke et al., 2010a; Holt, 2021). Therefore, more detailed study is required.

The results of this study shows that laying hens housed in enriched cages were exposed to a higher risk of occurrence of *S*. Enteritidis and *S*. Typhimurium (Table 3). Furthermore, it is evident that *Salmonella* strains of public health significance (*S*. Enteritidis, *S*. Typhimurium) were confirmed in four flocks (57.14%) of laying hens housed in enriched cages, and in one flock (9.09%) of laying hens housed in housing system with outdoor run. Methner et al. (2006) reported that the dominant serovar was *S*. Enteritidis, which was isolated in laying hen faeces in 78% of cases, which is why vaccination against this strain is recommended. Table 3 shows that in the present study, the highest percentage of flocks of caged housed laying hens was positive for *S*. Enteritidis and *S*. Typhimurium (28.57%), while S. Enteritidis was not isolated at all in the housing systems with outdoor runs. In housing

Table 3. Occurrence of *Salmonella* spp. in different housing systems of laying hens in the period from 2020 to 2023

Housing system	*Salmonella* spp.	No. positive samples/ flocks	% positive flocks of total flocks positive to *Salmonella* spp.
Enriched cages	S. Enteritidis	4 / 2	28.57
	S. Typhimurium	4 / 2	28.57
	S. Mbandaka	2 / 1	14.29
	S. Galiema	2 / 1	14.29
	S. Montevideo	2 / 1	14.29
Housing systems with closed outdoor runs	S. Typhimurium	2 / 1	9.09
	S. Coeln	12 / 4	36.36
	S. Mbandaka	2 / 1	9.09
	S. Salmonella II	2 / 1	9.09
	S. Veneziana	2 / 1	9.09
	S. Abony	2 / 1	9.09
	S. Kottbus	2 / 1	9.09
	S. Paratyphi B	2 / 1	9.09

systems with outdoor runs, *S*. Coeln was most often detected (36.36%). Gambi et al. (2022) studied the presence of subspecies and serotypes of *Salmonella* in the colon of wild carnivores (red fox, European badger, wolf). They isolated a total of 67 strains belonging to S. enterica subsp. enterica, S. salamae, S. diarizonae and S. houtenae. The most commonly isolated serotypes were *S*. Veneziana and *S*. Typhimurium, and additionally, in comparison with the present study, *S*. Agona, *S*. Coeln and *S*. Kottubus were isolated. This discovery further emphasises the importance of monitoring possible sources of infection of *Salmonella* for humans and domestic animals. On the other hand, environmental pollution with human waste can be associated with the presence of zoonotic serotypes of *Salmonella* in the wild (Afem and Sischo, 2016). Mollenhorst et al. (2005) confirmed a significantly lower prevalence of *S*. Enteritidis in laying hens housed in non-conventional cage systems in comparison with deep bedding systems. *S*. Typhimurium is more widely present in wild animals, pigs and cattle than in poultry, and Carrique-Mas and Davies (2008) stated that free range laying hens are exposed to a higher risk of infection with *S*. Typhimurium than flocks held in systems without outdoor runs. Chousalkar et al. (2016) confirmed that strains of *S*. Typhimurium isolated from the faeces of wild birds and foxes in the vicinity of free-range laying hen farms were also isolated from dust and the faeces of those laying hens, with reported cases of disease in humans caused by food. Therefore, environmental factors could have a significant role in the emergence of *S*. Typhimurium in free range laying hen housing. However, this was not confirmed in a study by EFSA (2007) or the study by Van Hoorebeke et al. (2010a). Housing laying hens in conventional cages has proven to be a significant risk factor for the incidence of *S*. Enteritidis and *S*. Typhimurium (Methner et al., 2006; EFSA, 2007; Namata et al., 2008; Huneau-Salaün et al., 2009).

Conclusion

The research results lead to the conclusion that there is a low likelihood that transition from enriched cage systems to alternative housing systems, with the implementation of strict biosecurity measures, would result in an increase in *Salmonella* infections in laying hen flocks. Moreover, it appears that serovars of public health concern such as *S*. Enteritidis and *S*. Typhimurium are more common in cage housing.

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Pojavnost *Salmonella* spp. u jatima različito držanih kokoši nesilica na području istočne Hrvatske u razdoblju od 2020. do 2023. godine

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Infekcije ljudi salmonelama često su povezane s konzumacijom zaraženih jaja i proizvoda od jaja. Stoga su poznavanje pojavnosti *Salmonella* spp. u jatima kokoši nesilica i čimbenika koji utječu na prisutnost salmonela na farmama od iznimne važnosti. U Europskoj uniji od 2012. godine zabranjeno je držanje kokoši nesilica u klasičnim (konvencionalnim) kavezima. Dopušteno je držanje samo u obogaćenim kavezima ili alternativnim sustavima držanja. Zabrana klasičnih kaveza poboljšala je dobrobit kokoši nesilica, ali je potaknula pitanje o štetnim posljedicama za širenje i pojavnost zaraznih bolesti u jatima nesilica. Cilj je ovog istraživanja bio analizirati pojavnost *Salmonella* spp. u jatima kokoši nesilica na području istočne Hrvatske u razdoblju od 2020. do 2023. godine te utječe li sustav držanja na njihovu pojavnost. U navedenom razdoblju u Laboratoriju za dijagnostiku Veterinarskog zavoda Vinkovci u okviru provedbe Nacionalnog programa kontrole salmoneloze i službenog uzorkovanja u Republici Hrvatskoj na prisutnost bakterija *Salmonella* spp. ukupno su pretražena 1.534 uzorka izmeta kokoši nesilica iz 191 jata (106 jata držanih u obogaćenim kavezima i 85 jata u sustavu držanja s ispustom). Izdvajanje, identifikacija i pripadnost serovarova salmonela provedeni su standardnom metodom HRN EN ISO 6579-1:2017/A1:2020. *Salmonella* spp. utvrđena je u izmetu 7 (6,60 %) jata kokoši nesilica držanih u obogaćenim kavezima i 11 (12,94 %) jata kokoši nesilica u sustavu držanja sa zatvorenim ispustom. Rezultati istraživanja pokazuju da nije bilo značajne razlike u ukupnoj pojavnosti salmonela između jata kokoši nesilica držanih u sustavu držanja s ispustom i obogaćenim kavezima. Najveći postotak pozitivnih jata utvrđen je 2020. godine u 11,11 % jata kokoši nesilica držanih u obogaćenim kavezima i 18,18 % jata kokoši nesilica držanih u sustavu držanja s ispustom. Promatrano po županijama, tijekom četverogodišnjeg razdoblja postotak pozitivnih jata bio je značajno veći (P<0,05) u Brodsko-posavskoj u odnosu na Osječko-baranjsku i Vukovarsko-srijemsku županiju. Rezultati istraživanja upućuju na to da je mala vjerojatnost kako će prelazak s obogaćenih kaveza na alternativne sustave držanja, uz strogo pridržavanje biosigurnosnih mjera, povećati pojavnost infekcija salmonelama u jatima kokoši nesilica.

Ključne riječi: *kokoši nesilice, Salmonella* spp., *sustav držanja, obogaćeni kavezi, alternativni sustavi držanja*