Occurrence of *Salmonella* spp. in eggs from backyard chicken flocks in Portugal and Romania- results of a preliminary study

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ABSTRACT

The aim of this study was to conduct a preliminary investigation on the occurrence of *Salmonella* spp. in eggs from chickens raised in backyards in Portugal and Romania. A lack of compliance with safety practices by chicken owners, was demonstrated, especially in Portugal, as 96% of the eggs were visibly dirty and 92.5% were stored at room temperature. In Romania the 202 analysed eggs were *Salmonella* free, whereas in Portugal six of the 200 eggs sampled were positive for *Salmonella* spp. (3%). A positive egg for *Salmonella* spp. was found in 10.7% of the 56 backyard flocks sampled in Portugal. One egg exhibited contamination both in the shell-membrane mixture and in its content, while in the remaining eggs, the pathogen was found either in the shell-membrane (n=2) or in the yolk and white mixture (n=3). The serotypes *S*. Typhimurium (with identical PFGE patterns) and *S*. Enteritidis were isolated from five eggs and one egg, respectively. Whilst *S*. Enteritidis was sensitive to the 14 antibiotics tested, *S*. Typhimurium isolates presented divergent antimicrobial resistant phenotypes and three were classified as multi-drug resistant.

Keywords: *S. enterica; S*. Typhimurium; henhouse; storage; consumer preferences; Multi-drug resistance (MDR)

1. Introduction

Foodborne illnesses are an important public health problem worldwide due to the mortality, morbidity and costs associated with investigations, surveillance, and ultimately the prevention of illness (WHO, 2015). In Europe, foodborne salmonellosis, with nontyphoid *Salmonella* serotypes, is the second most commonly reported zoonosis amongst member states, with 91,857 confirmed human cases, 16,556 reported hospitalizations and 119 deaths in 2018. Since 2013 no trend towards a decrease has been observed (EFSA & ECDC, 2019).

While many food products have been associated with *Salmonella enterica* contamination, namely poultry, beef, fish and vegetables, raw or undercooked eggs and egg related products were

identified as the most important source of foodborne *Salmonella* outbreaks (CDC, 2018; EFSA & ECDC, 2019).

The prevalence of *Salmonella* in commercial table eggs is low in most developed countries (Martelli & Davies, 2012). Only 23 table eggs of the 6,252 analysed (0.37%) in 2018 were *Salmonella* positive (EFSA & ECDC, 2019). However little information is available on backyard eggs and to our knowledge only one study was conducted recently in Europe (Fenollar, Domenech, Ferrus, & Jimenez-Belenguer, 2019)

The current shift in consumer preferences for products perceived as "more natural", "organic", "humanely-raised", and viewed as healthier, lead to an increased trend for the consumption of eggs from backyard raised chickens. Backyard farming, as a source of household food supply, is very popular in the rural areas of Portugal and Romania, and frequently consumers living in urban centres also pursue domestically grown or produced foods.

In a survey conducted across ten European countries, in the scope of the SafeConsume project (http://safeconsume.eu/), a larger number of respondents in Portugal and Romania (38.8% and 49.0%, respectively) indicated that they typically get the whole, raw eggs they eat at home from backyard hens (either their own or those of relatives, friends or even unknown people who sell eggs in front of their courtyard gates or in grey markets), in comparison to the respondents in Norway (4.3%), United Kingdom (5.7%), Germany (6.9%), Denmark (9.5%), France (15.6%), Hungary (16.6%), Spain (17.7%), or Greece (29.1%) (unpublished data). Therefore, the aim of this study was to conduct a preliminary investigation on the occurrence of *Salmonella* spp. in eggs from chickens raised in backyards in Portugal and Romania.

2. Material and Methods

2.1 Sampling

The present study was carried out in the North region of Portugal and South-East region of Romania, where the research laboratories are located. The counties Galati and Braila, from where most

of the samples have been taken in Romania, are Sentinel counties. In Portugal, most of the family farms are located in the North region. Participants, backyard egg producers, were recruited *via* the researchers' personal contacts and were asked to donate two eggs. A standardized questionnaire on eggs and chicken flocks production was given to each participant. Eggs were collected on two different occasions: Winter (December 2017-January 2018 - Christmas holidays, when traditional dishes and desserts prepared with eggs are very popular in both countries) and Spring/Summer (April-August 2018 – when most cases of human salmonellosis occurs) (ECDC, 2020). In Romania, 16 eggs were additionally bought in grey markets but from domestic production (Table 1). Eggs were collected by each flock owner and transported immediately to the laboratory in plastic bags or cardboard boxes. At the laboratories, eggs were stored at room temperature or at 4 ºC according to previous storage conditions in the collection place (Supplemental Tables 1 and 2) until further microbiological analysis, that was carried out in less than 48 h. A total of 402 eggs were analysed.

2.2. *Salmonella* spp. isolation

Presence of *Salmonella* was investigated both in the egg's internal contents (yolk and white mixture) and on the eggshell. Using alcohol-sterilized gloves, unwashed eggs were broken using an alcohol flame sterilized scalpel with a single strike in the centre. Eggs contents (whites and yolk) were separated from the shell, placed into a sterile stomacher bag and homogenised with 225 mL of buffered peptone water (BPW; Biokar Diagnostics, Beauvais, France) in a stomacher for 1 min. To prepare the eggshells samples, an adaptation of the shell crush methodology previously described by Musgrove et al. (2005) was applied instead of the eggshell surface wash procedure, as this methodology showed higher sensitivity in *Salmonella* recovery. The shell and membrane (and any adhering albumen) were crushed and mixed by hand in a double stomacher bag in a 1:10 dilution with BPW. After each egg sampling, the operator's gloves were disposed and replaced with new gloves to prevent cross-contamination between samples. The detection of *Salmonella* spp. was further carried out following the procedures established by the International Organization for Standardization (ISO), ISO 6579-1:2017 (ISO, 2017), using two selective enrichment broths, (i) Rappaport Vassiliadis soya peptone (RVS,

bioMérieux Hazelwood, Missouri, USA) and (ii) Muller–Kauffmann tetrathionate novobiocin (MKTTn, bioMérieux); xylose-lysine-deoxycholate (XLD; VWR, Darmstadt, Germany) agar was selected as the selective solid isolation media, and RAPID'Salmonella medium (Bio-Rad, Hercules, California, USA) as a second agar selective medium. Suspect colonies on selective plating media were streaked on non-selective agar medium (tryptic soy agar, TSA), incubated overnight at 37 ºC, and biochemical confirmation was performed using triple sugar iron agar (TSI agar) and urea agar (Christensen) according to the ISO 6579-1:2017 (ISO, 2017).

2.3. Confirmation and identification of *Salmonella* serotypes

Presumptive positive *Salmonella* colonies identified by phenotypical characteristics on TSI and urea agar were subjected to latex agglutination assay (Oxoid™ *Salmonella* Test Kit; Thermo Fisher Scientific, Indianapolis, USA) according to the manufacturer's instructions. Isolates confirmed as *Salmonella* spp. were further typed to serovar level at the Portuguese National Reference Laboratory *Instituto Nacional de Saúde Doutor Ricardo Jorge* according to the ISO/TR 6579-3:2014 (ISO, 2014).

2.4. Antimicrobial susceptibility testing

The antimicrobial susceptibility test was performed using the disk diffusion method according to The Clinical and Laboratory Standards Institute (CLSI, 2017). Briefly, a colony of each *Salmonella* isolate was suspended in sterile saline to obtain a turbidity equivalent to a 0.5 McFarland turbidity standard. Subsequently, a sterile cotton-tipped swab was dipped into the cell suspension and streaked onto a plate of Mueller-Hinton agar (MH; Biokar Diagnostics) in three directions. The plates were dried for ca. 5 min and discs containing the antibiotics (Oxoid, Hampshire, England) were aseptically placed on the agar surface. The following antimicrobials were tested: amoxicillin-clavulanic acid (20-10 μg), ampicillin (10 μg), ampicillin-sulbactam (10/10 μg), cefoxitin (30 μg), ceftazidime (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), ertapenem (10 μg), gentamicin (31 μg), imipenem (10 μg), meropenem (10 μg), nalidixic acid (30 μg), sulfamethoxazole- trimethoprim (10 μg), tetracycline (30 μg). *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were included as

controls in antimicrobials susceptibility assays. The plates were incubated at 37 ºC for 18 h and then the diameters of the zones of growth inhibition were measured. Results were evaluated according to breakpoints inhibitory zone diameter established by the CLSI (2017). Isolates exhibiting resistance to at least three structurally unrelated antibiotics were classified as multidrug resistant (Magiorakos et al. 2012).

2.5. Subtyping by Pulse-Field Gel Electrophoresis (PFGE)

Pulsed-field gel electrophoresis was performed according to the PulseNet standardized laboratory protocol for molecular subtyping of *Salmonella* (PulseNet, 2017). Restriction digestion of DNA in agarose plugs was carried out with the enzymes: *Xba*I, *Spe*I and *Avr*II (New England Biolabs Inc., Ipswich, Massachusetts, USA). Restricted plugs were loaded into a 1% SeaKem Gold agarose gel (Lonza Group AG, Basel, Switzerland) and submitted to electrophoresis in 0.5× TBE buffer at 14 °C for 19 h), at 6 V/cm and an included angle of 120º on a Chef DR III system (Bio-Rad). *Salmonella* serotype Braenderup H9812 plugs restricted with *Xba*I were used as the molecular size standard. Following the electrophoresis, gels were stained using ethidium bromide solution (MP Biomedicals, Santa Ana, California, USA) and photographed using Gel Doc XR+ System with Image Lab Software (Bio-Rad Laboratories). BioNumerics v.7.6.2 (Applied Maths, Sint-Martens-Latem, Belgium) was used for numerical analysis of the enzymes restriction patterns and Dice coefficient was used for similarity analysis (position tolerance of 1.5%). PFGE patterns were clustered using the Dice coefficient and the unweighted pair-group method using arithmetic averages (UPGMA).

3. Results and discussion

3.1. Occurrence of *Salmonella* spp. in backyard eggs in Portugal and Romania

Detailed information on backyards and eggs surveyed in Portugal and Romania is given in Supplemental Tables 1 and 2, respectively. In Romania the 202 eggs analysed were *Salmonella* negative, whereas in Portugal six of the 200 eggs yielded *Salmonella* spp. (3%). Interestingly, only one

of the two eggs analysed from each backyard and collected on the same date tested positive, and sampled flocks tested *Salmonella*-positive once, i.e. never in both seasons. Seven *Salmonella* isolates were recovered for further characterization.

A low number of eggs was collected which it makes impossible to reach reliable conclusions, it is important to highlight that (i) a higher number of eggs showing visibly dirty shells were collected in Portugal (96%) than in Romania (38.6%) and also (ii) that in Romania most of the eggs (86.6%) were kept refrigerated while in Portugal they were kept at room temperature (92.5%) (Table 1). According to Schoeni, Glass, McDermott, & Wong (1995) faeces on egg surfaces increased *Salmonella* growth up to 5 logs during storage at 25 ºC. Storage at cold temperatures is a critical factor to prevent *Salmonella* growth in egg's content. It has been demonstrated that in artificially contaminated eggs *Salmonella* is able not only to survive but also to rapidly multiply and achieve levels > 10^6 cells during storage at 25 ºC (Whiley & Ross, 2015).

Data on *Salmonella* contamination in backyard eggs is scarce and variable. Two studies have reported absence of *Salmonella* in backyard eggs analysed in Spain (n=10; Fenollar, Domenech, Ferrus, & Jimenez-Belenguer, 2019) and in Egypt (n= 200; Eid, Nasef, & Erfan, 2015), and one study in India showed a 10% occurrence (n= 40; Samanta et al., 2014). As in the present study, previous studies also analysed only a low number of backyard eggs making difficult a quantitative comparison with commercial table eggs. Nevertheless, taking into account these results, those on the occurrence of *Salmonella* in backyard chickens (e.g. Manning, Gole & Chousalkar, 2015) and the fact that *Salmonella* predominated as the leading cause of food-borne outbreaks in domestic settings in 2018 (63.4% of 287 outbreaks) (EFSA & ECDC, 2019) the risk posed by backyard eggs cannot be neglected and needs to be further investigated. In fact, a higher occurrence of *Salmonella* in backyard eggs than in commercial eggs may be anticipated considering the absence of preventive measures in the domestic situation that are applied in commercial laying chicken houses (e.g. biosecurity programmes, vaccination, hygiene practices regarding the laying houses). Additionally, domestic chickens are frequently raised with access to outdoors spaces and physical contact with other animals (e.g. farm animals, other birds) which can, hypothetically, contribute to increase the prevalence of *Salmonella*. In Portugal 42% of the 55

flocks surveyed were raised in a free-range system, and 29% were in contact with other animals, e.g. rabbits, turkeys, dogs or wild birds (Supplemental Table 1); five out of the six positive flocks were raised in free-range conditions. However, it must be pointed out that different studies aiming to compare caged housing versus cage-free egg production systems generated contradictory results and that the study conducted in India by Samanta et al. (2014) revealed feed and drinking water as a source of *Salmonella* spp. in backyard chickens. Thus, currently there is no consensus on which housing systems results in less *Salmonella* contamination (Whiley & Ross, 2015).

A higher number of positive samples was obtained in the Winter than in the Spring/Summer; i.e., eggs from five of the 50 domestic hens tested were positive (10%) in the Winter, while in the Spring/Summer, only one egg from one of the 50 henhouses surveyed was contaminated (2%). Previous studies reported a similar trend (Davies & Breslin, 2004; Suresh, Hatha, Sreenivasan, Sangeetha, & Lashmanaperumalsamy, 2006). Radkowski (2002) demonstrated that death rate of *Salmonella* in eggshells increases at higher temperatures (20 ºC or 30 ºC) than lower (2 ºC). Others have shown that *Salmonella* penetration rates through the eggshells pores rise at lower temperatures, due to a positive temperature differential that occurs when the egg is warmer than the environment. At high levels of moisture (e.g. eggs laid on moist surfaces during the rainy season; https://www.climatestotravel.com/climate/portugal), as bacterial cells have easier access to the egg's interior if they are introduced on the egg surface before the cuticle has sufficiently dried (Howard, O'Bryan, Crandall, & Ricke, 2012; Messens, Grijspeerdt, & Herman, 2005).

As the number of tested eggs and *Salmonella* positive eggs was low, future further studies should be performed to validate this trend using a larger sample size.

3.2. Contamination of eggshell and egg contents with *Salmonella* spp.

Of the six eggs positive for *Salmonella*, only one exhibited contamination both in the shell-membrane mixture and in the content (isolates SLM 1 and SLM 1C). In this situation, as the eggshell was not washed or sterilized, contamination from the shell to the egg content in the moment that the eggs were broken cannot be completely ruled out. However, it is important to point out that this egg

had been stored at room temperature for (at least) three weeks after laying. On the remaining eggs, the pathogen was found either on the shell-membrane (n=2; isolates SLM 27C and SLM 55C) or in the yolk and white mixture (n=3; isolates SLM 5, SLM 9 and SLM 7). Contamination of the egg's content, membranes or shell, may occur if the hens' reproductive tract is colonized with *Salmonella* spp. (Gantois et al., 2009). The eggshell can also become contaminated after oviposition via environmental contamination, i.e., through contact with contaminated faeces or surfaces. Penetration of bacteria from the egg surface into the egg core has been demonstrated (Gole et al., 2014; Messens, Grijspeerdt, & Herman, 2005). Whiley, Fallowfield, Ross, McEvoy & Hiley (2016) demonstrated that storage at refrigeration temperatures decreased the likelihood of *S*. Typhimurium penetration of the eggshell membrane and further contaminate the egg contents.

All the isolates from eggs collected in the Winter were identified as *S. enterica* serovar Typhimurium, while the single isolate recovered in the Summer was identified as *S. enterica* serovar Enteritidis (Table 2). Although *S*. Enteritidis is more common in commercial eggs, *S*. Typhimurium is commonly isolated from wild birds (Martín-Maldonado et al., 2019). *S*. Typhimurium, *S*. Enteritidis and other serotypes, are more frequently reported on eggs surfaces, but have also been recovered from the egg's contents (Martelli and Davies, 2012). It is also important to observe that although *S*. Enteritidis was the most common serovar recovered from patients until 2010 in Portugal, since then this trend is not observed and in 2018 and in the first semester of 2019 the number of *S*. Typhimurium and S. *Enteritidis* isolates was similar (Silveira, 2019).

3.3. Antimicrobial resistance and molecular typing of *Salmonella* spp. isolated from backyard eggs in Portugal

Molecular typing results, using PFGE, of the seven *Salmonella* spp. isolates are presented in Fig. 1. Macrorestriction analyses yielded two distinctly separate clusters, differentiating the single serotype Enteritidis strain from the serotype Typhimurium isolates. The six serotype Typhimurium isolates displayed identical PFGE patterns. The genetic relatedness among serotype Typhimurium isolates collected in different backyards could indicate either that they belong to a prevalent clone in

233 the region/specific niche, or that PFGE analysis using the three restriction enzymes was not sufficiently
234 powerful to differentiate genetic differences. To confirm this, it would be necessary to perform the
235 PFGE analysis with an increased number of macroestriction enzymes, as previously suggested, or to
236 use a combination of different typing methods (Zheng et al., 2011).

237 Different antimicrobial susceptibility phenotypes were observed (Table 3). The Enteritidis
238 isolate showed susceptibility to all the antibiotics tested, whilst all Typhimurium isolates were resistant
239 to ampicillin and chloramphenicol, and either intermediately or completely resistant to tetracycline.
240 Additionally, three Typhimurium isolates were classified as MDR (isolates SLM 1, SLM 1C, and SLM
241 55C), with three different resistance patterns found. Isolates SLM 1 and SLM 1C, isolated from the
242 same egg, but from the contents or shell, respectively, showed different resistance to tetracycline and
243 ampicillin-sulbactam combination (Table 3). The antimicrobial resistance phenotypes observed in this
244 study, are in agreement with the findings by previous researchers (e.g. Fernández Márquez et al., 2017).
245

246 **Conclusion**

247 The low number of eggs analysed is a major limitation of this study. Nevertheless it was demonstrated,
248 to our knowledge for the first time in an European country, that eggs from domestic chicken flocks can
249 be a source of MDR *Salmonella* – 10.7% of the 56 backyard flocks sampled had a positive result. It
250 was also found that risky practices are being undertaken by backyard eggs producers – lack of proper
251 hygiene and storage temperature. The need for further studies to evaluate the actual contribution of
252 consumption of backyard eggs as a vehicle of salmonellosis and environmental dissemination of
253 *Salmonella* serotypes other than Enteritidis should be highlighted since the consumers are being
254 exposed to eggs from uncontrolled origins, which can bias the outcome of the control measures applied
255 by health and food authorities. Development of specific programs to alert consumers about the risk of
256 consuming backyard eggs, particularly if these are raw or undercooked, would be crucial to support the
257 fight against *Salmonella* and to minimize the prevalence of salmonellosis. Vulnerable consumers
258 (pregnant, elderly, children, immunocompromised) should be informed on how to manage the risk e.g.
259 good management practices in backyard eggs production, eggs pasteurization, only use backyard eggs

in cooked dishes, refrigerate eggs immediately after laying or consume within two days if not stored refrigerated (<4 ºC).

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FIGURES CAPTIONS

Fig. 1. *Xba*I, *Spe*I and *Avr*II PFGE restriction patterns for seven *Salmonella* spp. isolated from backyard eggs

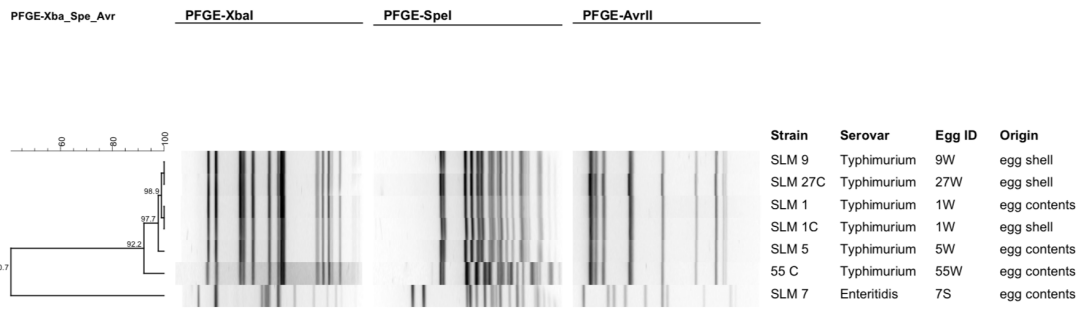

Table 1 Characterization of the eggs sampled

	Number of Backyards Sampled (Number of Eggs)					
	Winter and Spring/Summer	Winter	Spring/Summer	Total number of backyards (total number of eggs)	[c]Egg storage	Visual appearance
Portugal	44 (176)	6 (12)	6 (12)	56 (200)	RT:185 (92.5%) R:15 (7.5%)	Clean: 8 (4%) Dirty:192 (96%)
Romania	2 (8)	45 (89 [a])	47 (89 [a])	94 (202)[b]	RT:27 (13.4%) R: 175 (86.6%)	Clean: 124 (61.4%) Dirty:78 (38.6%)

[a] One of the backyards flocks' owners only donated one egg

[b] In Romania eggs were also collected from open markets (14 in the Winter and 2 in Spring/Summer)

[c]RT-Room Temperature; R-Refrigerated

Table 2. Results for eggs from henhouses with positive results for *Salmonella* spp.

Season	Hen house ID	Egg ID	White and Yolk mixture	Shell	Isolate code	Serovar
Winter	1	1W	Positive	Positive	SLM 1/SLM 1C	Typhimurium
	3	5W	Negative	Negative		
	5	9W	Positive	Negative	SLM 5	Typhimurium
	14	27W	Negative	Negative		
	28	55W	Positive	Negative	SLM 9	Typhimurium
	4	7S	Negative	Negative		
	1	1W	Negative	Positive	SLM 27C	Typhimurium
	3	5W	Negative	Negative		
	5	9W	Negative	Positive	SLM 55C	Typhimurium
	14	27W	Negative	Negative		
Summer	28	55W	Positive	Negative	SLM 7	Enteritidis
	4	7S	Negative	Negative		

Table 3. Antimicrobial susceptibility patterns among seven *Salmonella* spp. recovered from backyard eggs determined *via* disk diffusion procedure in accordance with CLSI standards (CLSI, 217).

Antimicrobial agent	Disk Content, μg	*Salmonella* spp. isolate[a]						
		SLM 1[b]	SLM 1C[b]	SLM 5	SLM 9	SLM 27C	SLM 55C	SLM 7[b]
PENICILLINS								
Ampicillin	10	R	R	R	R	R	R	S
ß-LACTAM/ß-LACTAMASE INHIBITOR COMBINATIONS:								
Amoxicillin-clavulanic acid	20/10	I	I	I	S	S	S	S
Ampicillin-sulbactam	10/10	R	I	S	S	I	I	S
CEPHEMS								
Cefoxitin	30	S	S	S	S	S	R	S
Ceftazidime	30	S	S	S	S	S	S	S
CARBAPENEMS								
Ertapenem	10	S	S	S	S	S	S	S
Imipenem	10	S	S	S	S	S	R	S
Meropenem	10	S	S	S	S	S	S	S
AMINOGLUCOSIDES								
Gentamicin	10	S	S	S	S	S	S	S
TETRACYCLINES								
Tetracycline	30	I	R	I	I	I	I	S
FLUORQUINOLONES								
Ciprofloxacin	5	I	S	S	S	S	S	S
QUINOLONES								
Nalidixic acid	30	S	S	S	S	S	S	S

FOLATE PATHWAY INHIBITORS:								
Sulfamethoxazole- trimethoprim	23.75/1.25	S	S	S	S	S	S	S
PHENICOLS								
Chloramphenicol	30	R	R	R	R	R	R	S

[a] Isolates were categorized as resistant (R), intermediate (I) or sensitive (S) to each antimicrobial using the inhibitory zone diameter breakpoints recommended by the CLSI (2017).

[b] Isolates classified as multidrug resistant (MDR) strain, i.e. if exhibiting resistance to at least three structurally unrelated antibiotics, according to Magiorakos et al. (2012).