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Determination of the within and between flock prevalence and identification of risk factors for *Salmonella* infections in laying hen flocks housed in conventional and alternative systems

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ABSTRACT

Salmonella outbreaks in humans are often linked with the consumption of contaminated eggs. Therefore a profound knowledge of the actual prevalence of *Salmonella* spp. in laying hens and the factors that influence the presence and persistence of *Salmonella* on a farm is of utmost importance. The housing of laying hens in conventional battery cages will be forbidden in the European Union (EU) from 2012 onwards. There is an urgent need to evaluate whether this move to alternative housing systems will influence the prevalence of *Salmonella* in laying hens. Therefore, a cross-sectional study was performed in 5 European countries (Belgium, Germany, Greece, Italy and Switzerland) to determine the between and within flock prevalence of hens shedding *Salmonella* and to investigate whether there is an effect of the housing type on *Salmonella* prevalence. In total 292 laying hen farms were sampled in the month prior to depopulation. An on-farm questionnaire was used to collect information on general management practices and specific characteristics of the sampled flock. Twenty-nine flocks were found positive for at least 1 *Salmonella*-serotype. In these flocks the within flock prevalence of shedding hens, determined by individual sampling of 40 hens, varied between 0% and 27.50%. A wide variety of serotypes was isolated with *Salmonella* Enteritidis as the most common. Housing in conventional battery cages, the absence of dry cleaning in between production rounds and sampling in winter turned out to be risk factors for the shedding of *Salmonella* Enteritidis or Typhimurium ($P < 0.05$).

1. Introduction

Already since the late 1960s there was a growing public awareness concerning farm animal welfare which generally resulted in a consumer's aversion to eggs produced by laying hens housed in cages (Appleby, 2003). This finally led to a ban of the housing of laying hens in conventional

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battery cages in the EU, from January 1st 2012 onwards (Council Directive 1999/74/EC). From this date on, the housing of laying hens in the EU will be restricted to enriched cages and alternative systems. According to the legislation laying hens housed in enriched cages must have at least 750 cm^2 of floor space per hen, a nest, perches and litter. These enriched cages exist in a wide variety of group sizes (EFSA, 2005). The so-called alternative systems are non-cage systems consisting of an indoor area either or not combined with outdoor facilities (EFSA, 2005; LayWel, 2006). The outdoor run may be covered ('wintergarden') or uncovered ('free-range'). Two main categories can be distinguished: single level systems where the ground floor is fully or partially covered with litter and aviaries with a ground floor area plus one or more platforms (EFSA, 2005; LayWel, 2006). The above mentioned ban on conventional battery cages aims to improve the welfare of laying hens (Wall et al., 2004). Yet it has also initiated the question whether there are not any adverse consequences of this decision on the spread and/or persistence of infectious diseases in a flock. This fear is based on the opinion that one of the big advantages of conventional battery cages is that, because hens are separated from their faeces, the risk for disease transmission through faeces can be minimized (Duncan, 2000). As an example, in Switzerland an increase in the incidence of bacterial infections in chicks and laying hens could be seen in a 12-year period after the Swiss ban of conventional battery cages (Kaufmann-Bart and Hoop, 2009). This leads to the question whether the same effect is to be expected for zoonotic pathogens such as *Salmonella* or *Campylobacter*. After all *Salmonella* is worldwide still an important cause of human disease (EFSA, 2009). In Europe, *Salmonella* Enteritidis and *Salmonella* Typhimurium are the most commonly isolated serotypes in human cases of salmonellosis (WHO, 2006; EFSA, 2007) and contaminated eggs still remain the most important source of infection with *Salmonella* Enteritidis for humans (Crespo et al., 2005; De Jong and Ekdahl, 2006; Delmas et al., 2006).

The EU ban on conventional battery cages combined with high EU *Salmonella* prevalence data, indicated by the results of the European Food Safety Authority (EFSA) baseline study on the prevalence of *Salmonella* in European laying hen flocks (EFSA, 2007) urged the European Commission to call for a specific targeted research project to analyze and control egg contamination by *Salmonella* after the move of laying hens to enriched cages and alternative housing systems (the Safehouse-project). One of the main tasks was a cross-sectional field study carried out in 5 European countries (Belgium, Germany, Greece, Italy and Switzerland).

The aim of this paper was to determine the between and within flock prevalence of *Salmonella* spp. in laying hen flocks housed in conventional battery cage and alternative non-cage housing systems and to identify risk factors for the presence of *Salmonella* on laying hen farms.

2. Materials and methods

2.1. Selection of the sampled farms

Flocks were selected based upon a list of contact addresses of registered laying hen farms provided by the official Identification & Registration authorities. Only farms with a farm size of >1000 hens could be selected. Also the housing system was an important selection criterion. The composition of the subset of sampled farms that was aimed at was 1/5 conventional battery cage farms and 4/5 non-cage housing systems. Within these non-cage housing systems an equal presence of aviaries, floor-raised, free-range and organic farms was strived at.

The farmers were contacted by telephone. The purpose of the study was explained and 100% anonymity was guaranteed (except for Germany), the foreseen date of depopulation was marked to make sure that the farm could be sampled in the month prior to depopulation. The aim was to sample in total 340 farms. Since not all of the contacted farmers volunteered to participate and because of logistic reasons, this number was not entirely reached. In the final study 292 flocks from 292 different farms were sampled in Belgium ($n = 69$), Germany ($n = 84$), Greece ($n = 10$), Italy ($n = 30$) and Switzerland ($n = 99$), comprising conventional battery cage flocks and four types of non-battery cage housing systems (Table 1). On farms with more than one house, only one house was randomly selected to be sampled. When several flocks were present in a given house, only one flock was selected at random.

2.2. Moment of sampling

Participating laying hen farms were sampled 1 month prior to depopulation. The samplings were performed during a 19-month period from January 2007 to August 2008.

2.3. Number and sample type taken on-farm

On-farm sampling consisted of 5 pooled faeces samples, 40 cloaca swabs of 40 laying hens and 1 sample of 200 red mites. Depending on the housing system, the pooled faeces samples were taken as follows:

- Conventional battery cage flocks and aviaries: 5 samples of mixed fresh faeces were taken from dropping belts and scrapers. The farmer was asked to operate the manure belts of each stack. In this way fresh piles of faeces could be picked up from each level of manure belts and of cages both at the right and the left side of the stack and along the entire length of stack. Each pooled faeces sample consisted of approximately 250 g.
- Floor-raised, free-range and organic farms: for each of the 5 pooled faeces samples 60 piles of fresh faeces were

Table 1
Numbers and types of laying hen farms that have been sampled.

Country	Conventional battery cage	Floor-raised	Free-range	Organic	Aviary
Belgium	18	21	21	8	1
Germany	26	20	24	14	–
Italy	12	10	8	–	–
Greece	3	7	–	–	–
Switzerland	–	16	33	14	36
Total	59	74	86	36	37

collected from the floor and the slats. Each pooled faeces sample consisted of approximately 250 g.

The pooled faeces samples were placed in a sterile recipient. Gloves were changed in between collection of each pooled faeces sample. The red mites were collected wherever in the house they were available. Using a disinfected brush they were swept into a sterile recipient.

The cloaca swabs were taken from hens that could be caught without causing too much agitation. Care was taken that hens were selected evenly throughout the house. Forty hens in the flock were selected and from each hen a cloaca swab was taken by inserting a cotton-tipped swab approximately 5 cm into the cloaca, taking care to avoid contact with the surrounding feathers and skin. Afterwards, the swabs were placed in tubes containing Ames medium. The sample size of 40 swabs was calculated in function of an expected within-flock prevalence of 10%, an accepted error of 10%, a confidence level of 95% and a flock size of 10,000 (WinEpiscope 2.0). All samples were placed in an appropriate leak-proof bag and outer container and transported to the lab under ambient conditions and were incubated for bacteriological analysis (see further). Bacteriological analysis started on the day of sampling. If not, samples were stored at 4 °C for no more than 24 h.

2.4. Bacteriological analysis of samples

All samples were analyzed using ISO 6579:2002_Amd1:2007, as recommended by the Community Reference Laboratory for *Salmonella* in Bilthoven, The Netherlands for the detection of *Salmonella* in animal and environmental samples from primary production. From each pooled faeces sample, 25 g was added to 225 ml of buffered peptone water (BPW) (Oxoid, Basingstoke, Hampshire, UK). Each cloacal swab was placed in 9 ml of BPW. Pooled faeces samples were mixed in a stomacher bag for 1 min. All samples were incubated for 18 ± 2 h at 37 ± 1 °C. Next, 3 droplets of the pre-enrichment culture were inoculated onto a modified semi-solid Rappaport-Vassiliadis (MSRV) (Difco; Becton Dickinson) agar plate containing 0.01 g l^{-1} novobiocine and incubated for $2 \times 24 \pm 3$ h at 41.5 ± 1 °C. Suspect white culture from the border of the growth zone was plated on Brilliant Green Agar (BGA; Oxoid) and Xylose Lysine Deoxycholate agar (XLD; Oxoid), followed by incubation for 24 ± 3 h at 37 ± 1 °C. Presumed *Salmonella* colonies on BGA and XLD were biochemically confirmed using ureum agar, triple sugar iron agar and lysine-decarboxylase broth. Serotyping of *Salmonella*-isolates according to the Kauffmann-White scheme was performed in the national Reference Labs for *Salmonella* in each participating country.

2.5. Questionnaire design

The questionnaire was filled in during an on-farm interview on the same occasion of the collection of the samples. Questions related to general farm and flock characteristics (e.g. flock size, breed, age of the hens, medical treatments...) and biosecurity measures. Special attention was paid to the system in which the sampled flock was housed. A summary of the main items included in the questionnaire is given in Table 2. The same questionnaire was used in all participating countries. Before use the questionnaire was tested on 2 laying hen farms, one with conventional battery cages and one with a free-range production system to check whether the questions were relevant to the aim of this study.

2.6. Data processing and analysis

Information from the questionnaires was coded and put in a database (Excel, Microsoft Cooperation). Data were analyzed using SPSS 16.0 for Windows (SPSS Inc., Chicago, IL) and MLwiN (MLwiN version 2.0, Centre for Multilevel Modelling, Institute of Education, London, UK). One-way analysis of variance was used to examine differences in

Table 2
Summary of the main items included in the questionnaire to identify risk factors for *Salmonella* in laying hen farms (for continuous variables: median; for categorical variables: proportions) (total number of questions = 92).

	All flocks	Salm.[a] positive flocks	Salm.[a] negative flocks
Farm characteristics			
Total number of hens	9700.0	57500.0	9000.0
Number of poultry houses	1.0	1.5	1.0
Other animal production present (% of the farms)	48.3	22.7	50.4
Control of pests (% of the farms)	91.0	90.1	91.1
House and management characteristics			
Age of building (in years)	21.0	38.0	19.0
Age of infrastructure	9.0	21.0	9.0
Number of flocks present	1.0	1.5	1.0
Access to outdoor run (% of the farms)	63.4	22.3	66.7
Number of egg collections per day	2.0	1.0	2.0
Dry cleaning status (% of the flocks)	82.2	31.8	86.3
Sampled flock characteristics			
Number of hens in the flock	4820	22000.0	4798.0
Cumulative mortality in the flock since onset (in %)	8.00	7.00	8.00
Age of the hens (in weeks)	71.0	73.0	71.0
Salmonella vaccination status (% of the flocks)	28.1	13.6	29.3
Medical treatment (% of the flocks)	9.2	18.2	8.5

[a] *Salmonella* Enteritidis and/or Typhimurium.

farm and flock size and age of the infrastructure between the different housing systems. For conventional battery cages this was the number of years since the current cages were installed. For the other housing systems this was the number of years since the current equipment (nest boxes, perches, slats...) was installed in the sampled house.

The within flock prevalence of excretion for each flock positive for *Salmonella* Enteritidis or Typhimurium was calculated based on the 40 individual cloacal swabs. The 95% confidence interval (CI) was calculated as the prevalence $\pm 1.96\ SD/\sqrt{n}$ with SD being the standard deviation and n the sample size. For positive flocks with 0 positive cloaca swabs (only positive in pooled faeces) the maximum possible prevalence that could be missed due to coincidence was calculated using following formula: $D = (1 - (1 - CL)^{1/n}(N - ((n-1)/2))$ (WinEpiscope 2.0). With D = maximum number of positive animals missed due to coincidence, CL = the confidence level, N = population size and n = sample size.

The relationship between risk factors and *Salmonella* Enteritidis or Typhimurium status of the sampled farm was evaluated by means of a multilevel logistic regression model with the *Salmonella* Enteritidis/Typhimurium status of the sampled flock as a binary outcome variable. For this a flock was defined as being infected if at least one of the collected samples was positive for *Salmonella* Enteritidis or Typhimurium. The decision to take this outcome variable is based on the fact that these two serovars are the two most common isolated serovars in case of human salmonellosis (EFSA, 2009). To check the potential effect of the housing system, the following categories were used: conventional battery cages, indoor production (which means aviaries and floor-raised systems), free-range systems and free-range organic systems.

In a first step, all potential risk factors were tested univariably and only variables with a P-value < 0.2 were selected. The shape of the relationship with the outcome variable was assessed for all continuous variables by plotting the log odds of the outcome versus the continuous variable (Parkin et al., 2005). If there was a non-linear relationship, the continuous variable was categorized.

Next, a multivariable logistic regression model was built, including all variables with a univariable P-value < 0.20. Correlations between the selected independent variables (Pearson's and Spearman's ρ correlations) were determined. If two variables were highly correlated (this means with an r^2-value above 0.60), only the variable with the smallest P-value was included in the final multivariable model.

The distribution of the housing types within the different countries could possibly influence the risk factor analysis, because of clustering or non-independence of data. To address this, the analyses were performed using MLwiN. Hereby, 'country' was taken into account as a random effect. To evaluate the presence of confounding, a Mantel–Haensel analysis was used. Confounding was considered potentially significant when changes in the OR of more than 20% could be observed (Dohoo et al., 2003).

In the final multilevel logistic regression model, all two-way interactions between significant variables were evaluated (with the significance level set at $P < 0.05$). Odds ratios (OR), including 95% confidence intervals (CI), are reported for all significant variables.

3. Results

A total of 292 commercial laying hen farms have been sampled in Belgium, Germany, Greece, Italy and Switzerland. A detailed overview of the numbers and types of farms is presented in Table 1. The participation rate was more than 90% in Belgium, Greece, Italy and Switzerland and 70% in Germany. A description of the main characteristics of the sampled farms is presented in Table 3. The mean age of the sampled hens was 72.1 weeks, ranging from 45 to 121 weeks. Because of practical reasons 1 Greek flock was sampled halfway the production cycle, at 38 weeks of age. The size of the selected flocks varied between 480 and 96,000 hens. The number of laying hens on conventional battery cage farms was significantly higher than the number of hens on farms with a non-cage housing system ($P < 0.05$). The age of the infrastructure in conventional battery cage systems was significantly higher than on floor-raised, free-range and organic farms ($P < 0.05$). No significant difference in the number of houses on the farm could be seen between the different housing systems.

Of the 292 sampled farms, 29 were found positive for *Salmonella* in at least one of the samples taken. The between flock prevalences differed significantly ($P < 0.05$) between the countries: 1.43% (0.00–3.73%) (Belgium), 20.00% (11.68–28.72%) (Germany), 20.00% (0.00–44.58%) (Greece) and 30.00% (13.81–46.19%) (Italy). In none of the 99 sampled Swiss farms *Salmonella* could be found: 0.00% (0.00–3.70%). When looking only at *Salmonella* Enteritidis or Typhimurium, 22 flocks were found positive with

Table 3
Main characteristics of the 292 sampled farms.

	Median	St. dev.	Minimum	Maximum
Hens on the farm				
Battery	55,500	88360.6	2500	540,000
Aviary	4996	4849.9	1760	23,600
Floor-raised	13,100	48333.7	1000	304,000
Free-range	9000	15002.8	1000	80,000
Organic	2845	2916.5	1000	12,000
Hens in the flock				
Battery	24,300	18394.6	1000	96,000
Aviary	2537	1785.3	480	10,242
Floor-raised	6500	5652.4	950	28,500
Free-range	4361	6133.1	1000	28,000
Organic	1862	1820.4	1000	7000
Age of the production system (years)				
Battery	14.0	12.0	1.0	46.0
Aviary	16.0	7.1	1.0	33.0
Floor-raised	8.0	10.5	1.0	45.0
Free-range	7.0	7.9	1.0	43.0
Organic	8.0	5.3	1.0	20.0
Age hens (weeks)				
Battery	74.0	11.6	45	121
Aviary	67.0	9.8	60	97
Floor-raised	70.0	10.7	38	112
Free-range	70.0	7.0	49	90
Organic	68.0	6.1	62	84

Table 4
Detailed overview of the 29 laying hen flocks found positive for *Salmonella*.

Country	Housing type	*N* positive/sample type	Within flock prevalence (95% CI)	Serotype and phage type
Belgium	Conv. Batt.	5/5 faeces, 0/40 swabs	0% (0.00–7.21%)	*S.* Enteritidis PT 4a
Germany	Conv. Batt.	5/5 faeces, 0/40 swabs	0% (0.00–7.21%)	*S.* Enteritidis PT 21c, RDNC
Germany	Conv. Batt.	3/5 faeces, 0/40 swabs	0% (0.00–7.21%)	*S.* Enteritidis PT 4
Germany	Conv. Batt.	1/5 faeces, 0/40 swabs	0% (0.00–7.21%)	*S.* Enteritidis PT 4
Germany	Conv. Batt.	1/5 faeces, 0/40 swabs	0% (0.00–7.21%)	*S.* Enteritidis PT 4
Germany	Conv. Batt.	1/5 faeces, 2/40 swabs	5.00% (0.00–11.65%)	*S.* Enteritidis PT 8
Germany	Conv. Batt.	1/5 faeces, 0/40 swabs	0% (0.00–7.21%)	*S.* Enteritidis PT 4
Germany	Conv. Batt.	1/5 faeces, 0/40 swabs	0% (0.00–7.21%)	*S.* Enteritidis PT 4
Germany	Conv. Batt.	1/5 faeces, 0/40 swabs	0% (0.00–7.18%)	*S.* Enteritidis PT 8
Germany	Conv. Batt.	2/5 faeces, 1/40 swabs	2.50% (0.00–7.33%)	*S.* Enteritidis PT 4
Germany	Conv. Batt.	2/5 faeces, 0/40 swabs	0% (0.00–7.33%)	*S.* Enteritidis PT 4
Germany	Floor-raised	3/5 faeces, 4/40 swabs	10.00% (0.73–19.27%)	*S.* Enteritidis PT 4
Germany	Floor-raised	3/5 faeces, 1/40 swabs	2.50% (0.00–7.33%)	*S.* Enteritidis PT 8
Germany	Floor-raised	3/5 faeces, 0/40 swabs	0% (0.00–7.21%)	*S.* Enteritidis PT 8, *S.* subsp. I
Germany	Free-range	1/5 faeces, 0/40 swabs	0% (0.00–7.10%)	*S.* Enteritidis PT 4
Germany	Free-range	5/5 faeces, 11/40 swabs	27.5% (13.94–41.06%)	*S.* Enteritidis PT 4
Germany	Free-range	1/5 faeces, 0/40 swabs	0% (0.00–7.18%)	*S.* Enteritidis PT 4
Germany	Organic	1/5 faeces, 0/40 swabs	0% (0.00–7.16%)	*S.* Enteritidis PT 4
Greece	Floor-raised	5/5 faeces, 0/40 swabs	0% (0.00–7.21%)	*S.* Heidelberg
Greece	Floor-raised	5/5 faeces, 0/40 swabs	0% (0.00–7.18%)	*S.* Heidelberg
Italy	Conv. Batt.	2/5 faeces, 0/40 swabs	0% (0.00–7.21%)	*S.* Napoli, *S. enterica* subsp. *ent.*
Italy	Conv. Batt.	3/5 faeces, 0/40 swabs	0% (0.00–7.21%)	*S.* Virchow
Italy	Conv. Batt.	4/5 faeces, 0/40 swabs	0% (0.00–7.21%)	*S.* Enteritidis PT 27, NT
Italy	Conv. Batt.	0/5 faeces, 2/40 swabs	5.00% (0.00–11.75%)	*S.* Enteritidis, *S.* Bredeney
Italy	Conv. Batt.	0/5 faeces, 1/40 swabs	2.50% (0.00–7.34%)	*S.* Typhimurium NT
Italy	Conv. Batt.	1/5 faeces, 0/40 swabs	0% (0.00–7.21%)	*S. enterica* subsp. *enterica* NT
Italy	Floor-raised	3/5 faeces, 1/40 swabs	2.50% (0.00–7.33%)	*S.* Bareilly, *S.* Thompson
Italy	Free-range	2/5 faeces, 0/40 swabs	0% (0.00–7.21%)	*S.* Kottbus
Italy	Free-range	3/5 faeces, 1/40 swabs	2.50% (0.00–7.33%)	*S.* Enteritidis PT 14, NT

respective between flock prevalences of 1.43% (0.00–3.73%) (Belgium), 20.00% (11.68–28.72%) (Germany), 0.00% (0.00–2.40%) (Greece) and 13.30% (1.32–25.34%) (Italy).

Only 7 of the 29 positive farms were found positive both in the pooled faeces samples and in the cloacal swabs. Twenty flocks were only positive in the pooled faeces samples and 2 flocks were positive in one or more cloacal swabs but not in the pooled faeces samples. The number of positive pooled faeces samples ranged from 1 to 5. In the 9 flocks where positive cloacal swabs were found the within-

Table 5
Results of the univariable and multivariate analysis for the identification of risk factors for *Salmonella* Enteritidis or Typhimurium infection on 292 European laying hen farms.

Continuous variables		Univariable analysis		Multivariable analysis		
		OR	*P*-value	OR	95% CI for OR	*P*-value
Age of the infrastructure in years		1.07	<0.01	/	/	/
Number of flocks in the sampled house		1.39	0.04	/	/	/
Number of egg collections per day		0.19	<0.01	/	/	/
Categorical variable	***n***	**OR**	***P*-value**	**OR**	**95% CI for OR**	***P*-value**
Dry cleaning						
No	52	13.49	<0.01	14.37	4.54–45.51	<0.01
Yes (ref)	240	–	–	–	–	–
Vaccination status against *Salmonella*						
No	210	2.62	0.13	/	/	/
Yes (ref)	82	–	–	/	/	/
Type of housing			<0.01			<0.01
Conventional battery (ref)	59	–	–	–	–	–
Indoor production	111	0.09	<0.01	0.05	0.01–0.24	<0.01
Free-range	86	0.16	<0.01	0.18	0.05–0.73	0.02
Organic	36	0.09	0.02	0.17	0.02–1.73	0.13
Season of sampling			0.02			0.01
Winter (ref)	49	–	–	–	–	–
Spring	80	0.30	0.04	0.16	0.04–0.73	0.02
Summer	97	0.09	<0.01	0.06	0.01–0.40	<0.01
Autumn	66	0.44	0.15	0.64	0.16–2.60	0.53

flock prevalence varied between 2.50% and 27.50%. In the 20 flocks with positive pooled faeces but no positive cloacal swabs the maximum prevalence of hens shedding *Salmonella* that could have been missed due to coincidence was 7.33%. A detailed overview of the results of the bacteriological analysis with the within flock prevalences is presented in Table 4. The number of positive swabs was not significantly associated with the housing type, nor was the number of positive pooled faeces samples. On 214 of the 292 sampled farms red mites could be collected. None of the red mites samples was found positive for *Salmonella*.

For the identification of risk factors only *Salmonella* Enteritidis and Typhimurium and the following housing types were taken into account: conventional battery cages, indoor production systems, free-range and free-range organic systems. The factors associated with detection of *Salmonella* in the univariable analysis are listed in Table 5. In the final multivariable logistic regression model (Table 5) the absence of dry cleaning ($P < 0.01$), the housing type ($P < 0.01$) and the season of sampling ($P = 0.01$) turned out to be risk factors for a *Salmonella* Enteritidis/Typhimurium-infection in laying hen flocks.

4. Discussion

The housing of laying hens in conventional battery cages turned out to be a significant risk factor for *Salmonella* Enteritidis and/or Typhimurium. This is in accordance with the results of the baseline study on the prevalence of *Salmonella* in laying hen flocks, both at the EU level (EFSA, 2007) and at the level of individual member states (Methner et al., 2006; Namata et al., 2008; Huneau-Salaün et al., 2009). The reasons why a higher prevalence of *Salmonella* is found in cage housed laying hen flocks is likely to be a combination of factors. First of all flock size may have an effect (Mollenhorst et al., 2005; EFSA, 2007; Carrique-Mas et al., 2009; Huneau-Salaün et al., 2009). As also demonstrated in this study, in cage farms flock sizes are in general larger in comparison to non-cage farms. Second, the number of years that the current infrastructure is in use could also play an important role. In this study the age of conventional battery cages was significantly higher than that of the floor-raised, free-range and organic systems. In the univariable analysis the age of the infrastructure was identified as a significant risk factor. The effect of the age of the infrastructure may be explained by the fact that the older the infrastructure, the more difficult it gets to achieve sufficient standards of cleaning due to the wear of the materials, both of the production system and of the building itself, especially when it is taken into account that the level of environmental contamination increases significantly during a production cycle (Wales et al., 2007). The importance of such latent environmental sources of infections should not be underestimated since Davies and Wray (1996) described the survival of *Salmonella* in empty poultry houses for a period of 12 months. This implies the risk of 'transfer' of an infection between successive production cycles.

The multivariate model shows that "age of the infrastructure" does not remain significant when combined with production type. This implies that this factor is not sufficient to explain the risk conventional housing systems pose to *Salmonella* Enteritidis infections, and thus that other factors related to the housing system are involved. One of these other factors might be the fact that it is more difficult to thoroughly clean and disinfect cage systems and therefore remainders from previous infections may be more difficult to eliminate (Davies and Breslin, 2003; EFSA, 2007).

Another proof of the importance of cleaning is given by the fact that absence of dry cleaning in between production rounds was found to be a significant risk factor for the presence of *Salmonella*. Dry cleaning is the mechanical removal of organic material (manure, dust, feed spills...) before the wet cleaning (using water) is carried out. Only 82.2% of the sampled farms carried out dry cleaning of the laying hen houses where for wet cleaning and disinfection this was 93.2% and 92.5%, respectively. The value of dry cleaning in between production rounds is two-fold: on one hand it is very useful to remove organic matter that can harbor *Salmonella*. On the other hand it contributes to a more efficient disinfection since the presence of considerable amounts of organic protective matter such as manure, dust and spilled feed has an adverse effect on the efficacy of disinfection (Davies and Breslin, 2003; Gradel et al., 2004).

In contrast to other studies (Bouwknegt et al., 2004; Mollenhorst et al., 2005; Namata et al., 2008) but in accordance with Wales et al. (2007), a seasonable effect could be observed. The odds of a *Salmonella* infection were significantly higher in flocks that were sampled in winter compared to flocks that were sampled in the other seasons of the year. This could be explained by the fact that in housing systems with an outdoor run the hens are kept inside due to wet and cold weather conditions. A high density of animals is a well-known risk factor for *Salmonella* (EFSA, 2007; Huneau-Salaün et al., 2009). Furthermore, the air quality in laying hen houses seems to be lower in winter (Ellen et al., 2000; Nimmermark et al., 2009). This can cause stress in the hens, leading them from a *Salmonella*-carrying state to a *Salmonella*-shedding state.

The estimates of the within flock prevalence based on the cloacal swabs were usually relatively low indicating that in general only a small percentage of birds in the positive flocks were shedding the bacterium. It needs to be stressed that the estimates obtained are an indication of the number of birds shedding *Salmonella*, and not necessarily an accurate indication of the number of birds infected with *Salmonella*. It is likely that in a substantial proportion of the birds sampled negative still some low level *Salmonella* infection may be present. In that case the negative sample is due to the fact that the birds were not shedding the bacteria at the moment of sampling or the used sampling technique was not sensitive enough to detect the limited shedding. The sometimes large difference between the prevalence of infected and shedding birds has recently been clearly demonstrated by Van Hoorebeke et al. (2009). This also holds for the between flock prevalence.

Compared to the results of Belgium [27.7% (22.1–33.9%)], Germany [24.2% (21.2–27.5%)], Greece [25.7% (20.5–31.6%)] and Italy [7.9% (5.9–10.5%)] in the EFSA baseline study on the between herd prevalence of

Salmonella Enteritidis and/or Typhimurium on laying hen holdings (EFSA, 2007), only in Belgium and Greece the prevalence detected in our study, was lower. However, these findings should be interpreted with care because the farms that were contacted to be sampled were selected on the base of the housing type and thus not random. In addition the sampling scheme used in this study is different from the one of the EFSA baseline study.

5. Conclusion

The results of this study illustrate that, despite the fact that in non-cage housing systems the chance of oro-faecal transmission of *Salmonella* is much higher than in conventional battery cage systems, no higher prevalence of *Salmonella* could be observed in flocks housed in these alternative systems. Several management and biosecurity measures such as strict cleaning and disinfection practices have been identified as protective factors to minimize the introduction and persistence of *Salmonella* on laying hen farms. In future, a close follow up of the evolution in time, both of the prevalence of *Salmonella* spp. in laying hen flocks housed in different housing systems and in the diversity of serovars isolated and their significance for public health, will be necessary.

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