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BROILER HEALTH STATUS HAS A MAJOR NEGATIVE IMPACT ON BROILER FLOCK CONTAMINATION WITH *CAMPYLOBACTER* SPP. IN LITHUANIA*

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Abstract

The objective of this study was to determine risk factors for *Campylobacter* infection in broiler flocks in Lithuania. Each broiler flock was tested for the contamination with *Campylobacter* spp., and various broiler farm, flock and abattoir as well as the weather-associated characteristics were analysed using the statistical package SPSS. Study revealed that 59.3% of the examined broiler flocks were contaminated with *Campylobacter* spp. Statistical analysis revealed that broiler flock contamination with *Campylobacter* was abattoir- and farm-dependent. Among a number of risk factors (e.g. the number of broiler houses at the farm, the type of ventilation system, the presence of the anteroom and boot security, etc.) identified, two broiler health-associated characteristics: (i) broiler age and (ii) the average weight per bird at abattoir had the highest impact on the increased prevalence of *Campylobacter* spp. in broilers. According to our results broiler health status has a major negative effect on broiler flock contamination with *Campylobacter*. Thus, it needs to be considered when improving control of *Campylobacter* spp. in broilers.

Key words: broiler, campylobacteriosis, *Campylobacter* spp., *C. jejuni*

Campylobacter spp. is an important bacterial pathogen causing gastro–intestinal infection to human. In 2014 a total of 236 851 confirmed cases of campylobacteriosis were reported from EU countries, and the notification rate was 71.0 cases per 100 000 population. In Lithuania, campylobacteriosis is one of the most prevalent foodborne zoonoses in humans with the notification rate of 40.2 cases per 100 000 population (EFSA, 2015). The two species *Campylobacter jejuni* and *Campylobacter coli* are frequently associated with human infections. Most human cases of

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Campylobacter are foodborne, and handling or consumption of undercooked chicken is considered to be a major risk factor (Kapperud et al., 1993; Rodrigues et al., 2001; Tam et al., 2009; Alves et al., 2012). *C. jejuni* and *C. coli* readily colonise the gut and the caecal contents of chickens with extremely high numbers of campylobacters (up to 10^8 c.f.u./g) are detected (Rosenquist et al., 2009). The contamination of poultry carcasses with *C. jejuni* and *C. coli* occurs during processing, and although methods to control the contamination at slaughter are available, they are limited by their practical application, permissibility under European Union food legislation or acceptability to consumers (Katsma et al., 2007). At slaughter, over 90% of the flocks may be colonised by thermophilic *Campylobacter* (Evans and Sayers, 2000; Stern et al., 2001). As high numbers of thermophilic *Campylobacter* may be present not only in the intestinal tract but also on feathers and the skin of broilers this bacteria can be found throughout the slaughter line. The contamination of broiler carcasses may occur after visceral breakage at evisceration (Rosenquist et al., 2006; Hue et al., 2010), during scalding, defeathering or via cross-contamination in the processing line (Allen et al., 2007).

Many risk factors associated with *Campylobacter* colonisation of broiler flocks have been identified. These include the age of the birds at sampling (Bouwknegt et al., 2004; Barrios et al., 2006), season (Bouwknegt et al., 2004; Ellis-Iversen et al., 2009), production type (Nather et al., 2009), the presence of other farm animals/cattle on or adjacent to the broiler farm (Bouwknegt et al., 2004; Ellis-Iversen et al., 2009), partial depopulation/staggered slaughter (Hald et al., 2000; Ellis-Iversen et al., 2009), multiple broiler houses on the farm (Bouwknegt et al., 2004; Guerin et al., 2007), flock size (Nather et al., 2009; Barrios et al., 2006), water from a private water source (Lyngstad et al., 2008), drinking water systems (Nather et al., 2009) and farm hygiene (Evans and Sayers, 2000; Hald et al., 2000). Several studies point out that general broiler health or specific diseases may also be a significant risk factor to the increased flock contamination with *Campylobacter* spp. (Bull et al., 2008; Lawes et al., 2012).

The identified risk factors may be production system or farm specific, and may also be dependent on the climatic conditions of the country where the study is undertaken. Therefore, risk factors analysis in different geographical locations are extremely important for the improved control of *Campylobacter* spp. in broiler farms worldwide. In the current study we aimed to analyse the effects of farm and flock characteristics on the prevalence of *Campylobacter* in a large number of slaughtered broiler flocks over one year period in Lithuania.

Material and methods

The two main abattoirs in Lithuania have been chosen for this survey with the target sample size of 81 slaughter batches of broilers slaughtered during a one year period. The sampling was randomized so that the abattoir, the sampling day and the slaughter batch of birds to be sampled on a given day were based on a random selection. The total number of batches to be sampled was stratified by a calendar month, therefore, about 6–7 slaughter batches were scheduled for sampling each month.

Sample and data collection

In total 81 flocks belonging to 16 different broiler farms were investigated in Lithuania from October 2012 till December of 2013. Ten broilers cloaca samples were taken from each broiler flock during the slaughtering process.

For the collection of data, a structured questionnaire related to general information was sent to the poultry companies contacted at the abattoir to obtain details on farm and house characteristics and second standardized questionnaire of health status of each flock was collected at the abattoir (Tables S1 and S2).

Table S1. Description of bird health-related conditions recorded for flocks

Condition	Definition
Ascites	Increased fluid in abdomen
Skin lesions	Inflammation or lesions on skin
Runts	Extensive discoloration (dark red/brown) of bird
Septicaemia	Severe lack of muscle tissue and/or underweight bird
Hock marks	Black/brown discoloration accompanied by ulceration of lower-leg (tarso-metatarsus) area
Pad burn	Black/brown discoloration accompanied by ulceration of pad area
Breast blisters	Subcutaneos fluid forming a blister over the sternum
Pericarditis	Inflammation, discoloration, and/or adhesions of heart tissue
Perosis and Green leg	Green/blue discoloration of leg areas
Subcutaneos pus	Bulged skin area with pus on inspection
Enlarged liver	Abnormal liver size
Necrotic foci	May be seen in the liver and other organs
Peritonitis/Perihepatitis	Inflammation, discoloration, and/or adhesions of gut cavity/peritoneum tissue Inflammation, discoloration, and/or adhesions of liver tissue

Postmortem veterinary inspectors reject carcasses from the poultry processing line unfit for the food based on a variety of bird disease/welfare indicators by using defined criteria (Table S1). Carcasses were taken off the line either pre- or post-evisceration, and if they were removed for one condition, they were not assessed for the other.

***Campylobacter* spp. isolation and identification**

Broiler cloacal samples were collected using sterile cotton swabs and directly plated on *Campylobacter* blood free medium base (mCCDA) (610130, Liofilchem, Italy) with mCCDA Selective Supplement (81037, Liofilchem, Italy). The samples were transferred to the laboratory in a refrigerated bag at 4°C and were analysed immediately. Plates were incubated in a micro-aerophilic atmosphere (5% oxygen, 10% carbon dioxide, 85% nitrogen), generated by Campygen (CN25; Oxoid, UK) at 37°C for 48 h. After incubation the colonies, suspected of being *Campylobacter,* were obtained from each plate and examined by microscopy and further purified on blood agar plates (610188, Liofilchem, Italy) supplemented with 5% laked horse blood (HBL100, E&O Laboratories, Scotland), and incubated at 37°C for 48 h in microaerophilic atmosphere. The purified isolates were subsequently stored at –80°C in Brain Heart Infusion Broth (BHI) (610008, Liofilchem, Italy) with 30% glycerol (REACHEM, Slovakia) until further use.

Table S2. Definitions of explanatory variables included in the analysis of broiler flock contamination with *Campylobacter* spp. The distribution of variables in 81 broiler flocks in period from October 2012 to December 2013 is provided

Definition of variables	Level	The number of flocks	
		Campylobacter-positive	*Campylobacter*-negative
1	2	3	4
Season	Spring	12	7
	Summer	8	7
	Autumn	16	12
	Winter	9	10
Temperature	–2.5 to 2.5	15	11
	7.5 to 15	14	17
	20–25	8	7
Humidity	≤63	26	25
	>63	19	11
Farm characteristics			
Age of house	Old	31	10
	New	6	3
Litter type	Peat	29	12
	Straw	0	2
	Sawdust	9	3
Ventilation system	Shelter	22	5
	Exhaust	0	2
	Transverse, Longitudinal	2	1
	Shelter and wall	9	3
	Shelter and rear	5	2
	Combined	0	2
	Rear wall	0	2
Windows	Yes	27	9
	No	11	8
Water supply	Nipples	38	17
	Bell	0	0
On-site chlorination of water	Yes	27	9
	No	11	8
Number of sheds	≤2	1	5
	>2	37	13
Are all sheds stocking the same birds	Yes		
45			
36			
	No	0	0
House characteristics			
House anteroom	Yes	38	14
	No	0	2
Boot biosecurity	Yes	38	14
	No	0	2

Table S2 – contd.

1	2	3	4
Flock reared according to quality scheme	Yes	45	36
	No	0	0
Downtime prior to placement date	≤14 days	31	13
	>14 days	7	3
Stocking density (kg/m^2)	≤39	38	16
	>39	0	0
Is final depopulation	Yes	31	10
	No	7	4
The mortality of birds	≤5%	36	13
	>5%	1	1
Health characteristics at slaughter			
Age at slaughter	36–39	8	7
	40–42	34	24
	>43	3	5
Average weight per bird at slaughter	≤2,1	41	18
	>2,1	4	18

Additionally, a selective enrichment procedure was performed for each cloacal sample to detect the low numbers of *Campylobacter* spp. For this purpose, swabs were placed into 10 ml modified Exeter broth, which was prepared from Bolton broth (CM985, Oxoid, England) with *Campylobacter* growth (SV61, Mast Diagnostics, Merseyside U.K) and enrichment (Exeter) (SV59 Mast Diagnostics, Merseyside UK) supplements, and 1% of the laked horse blood as described previously (Williams et al., 2012). Enrichment tubes were incubated microaerobically at 37°C for 48 h. After incubation, 10 µl of the broth was streaked onto mCCDA plates. The identification and the purification of *Campylobacter* isolates were further performed as described above.

DNA extraction and multiplex PCR

DNA isolation was carried out using Chelex-100 reagent (C7901-100G, Sigma, USA). A 250 µl of the10xTE buffer and a 1 µl loop of bacterial culture were added to an Eppendorf tube, mixed well, and centrifuged at a maximum speed for 5 min. The supernatant was removed from tube and 250 µl of the 5% Chelex-100 solution in 1xTE buffer was added, mixed well, and samples were heated at 56°C for 45 min. Finally, the samples were heated at 100°C for 15 min and centrifuged at 14000 rpm speed for 5 min. Supernatant was placed in a new tube and stored at –20°C.

Campylobacter isolates were identified to the species level by a modification of the method and primers described by Wang et al. (1992) and Katzav et al. (2008). The primers C412F and CampR2 amplified a 857 bp fragment which occurred in all *Campylobacter* spp. A 323-bp amplicon was generated for *C. jejuni* and a 126-bp

amplicon was generated for *C. coli* by using a mix of primers hybridizing to the *C. jejuni* (primers CJF and CJR) and the *C. coli* (primers CCF and CCR). Each PCR mixture contained 2.0 µl of 2mM deoxynucleotide triphosphates (dNTPs) mixture (Thermo Scientific, Lithuania), 2.5 µl of 10xPCR buffer, 2.5 µl of 25 mM $MgCl_2$, 0.25 µl of Maxima Hot-Start Taq DNA polymerase 5 U/µL (Thermo Scientific, Lithuania), 1.0 µl of a 100 µmol l^{-1} primer mixture containing C412F, CampR2, *C. jejuni* and *C. coli* primers (Thermo Scientific, Lithuania), 1.0 µl of chromosomal DNA and MiliQ water to a final volume of 25 µl. PCR products were analysed by gel electrophoresis: 11 µl of each PCR product was loaded onto 1.9% TopVision Agarose gel (Thermo Scientific, Lithuania) containing 6.5 µl of ethidium bromide solution. The gel was visualized using UV light. The GeneRuler™ 100 bp DNA Ladder (Thermo Scientific, Lithuania) was used as a molecular size marker.

Statistical analyses

Statistical analysis was performed using the SPSS (Ver.17.0, 2006; SPSS Inc., Chicago, IL, USA) statistical package. We examined different factor distribution between the *Campylobacter*-positive and *Campylobacter*-negative broiler flocks to determine the relationship between the broiler flock contamination with *Campylobacter* and individual factors present at the flock and farm level (Table S2). Following statistical criteria were used for the data analysis in this study:

1) Pearson $\chi 2$ compatibility criterion – expressed as a percentage. This criterion was used to calculate the effect of month, season, abattoir, ventilation system, windows, chlorination of water, anteroom, boot biosecurity, downtime prior to placement date, flock divided, age of house and the number of mortality.

2) Mann-Whitney Z criterion – expressed in an interval scale and distributed outside the normal distribution. This criterion was used to calculate the effect of temperature, humidity, number of sheds, slaughter age, average weight per bird at slaughter and pathologies.

3) Spearman's rank correlation coefficient – was used to calculate the relationship between the status of broiler flock contamination with *Campylobacter* and company where it was reared.

4) The binary logistic regression (method ENTER) was used for multiple comparison to identify risk factors, assigned to the risk factors groups, having the highest influence on broiler flock contamination with *Campylobacter* spp.

A statistically significant difference was considered (i.e, the relationship between the *Campylobacter* contamination and a specific factor is statistically significant) when any method used in the study showed a reliability of more than 95%. (i.e unless $P<0.05$). In this case, further calculations of two statistical hypotheses were performed:

H_0 – the absence of a statistically significant association between broiler flock contamination with *Campylobacter* and a specific factor (this hypothesis is accepted if $P>0.05$).

H_A – the presence of a statistically significant association between broiler flock contamination with *Campylobacter* and a specific factor (this hypothesis is accepted if $P<0.05$).

Results

Out of 81 broiler flocks tested, 48 (59.3%) were positive for *Campylobacter* spp. (Table 1). *C. jejuni* was detected in 44.4%, *C. coli* in 2.5% examined broiler flocks. 9.9% of the flocks harboured both species at a time.

Table 1. Broiler flocks contamination with *Campylobacter* spp. at the abattoirs (n=81)

Campylobacter status and related species	% of flocks
Negative	40.7
C. jejuni only	44.4
C. coli only	2.5
C. jejuni and *C. coli*	9.9
Other *Campylobacter* spp.	2.5

The effect of the abattoir and weather on *Campylobacter* status of the broiler flock

Statistical analysis showed that significantly higher number of *Campylobacter* positive broiler flocks were slaughtered at the abattoir I in comparison to the abattoir II ($P<0.01$) (Table 2). This finding may indicate that specific slaughtering procedures used at the abattoir I could have an increased risk for the broiler flock contamination with *Campylobacter* spp. during slaughtering.

Table 2. Effect of season and on-farm factors on broiler flocks contamination with *Campylobacter* based on Pearson χ^2 compatibility criteria

	The number of flocks	*Campylobacter*-positive flocks (%)	*Campylobacter*-negative flocks (%)	χ^2	P
1	2	3	4	5	6
		Month			
January	5	80.0	20.0	21.225	0.031
February	8	22.2	77.8		
March	7	85.7	14.3		
April	5	75	25		
May	7	37.5	62.5		
June	9	22.2	77.8		
July	5	100.0	0.0		
August	1	100.0	0.0		
September	5	80.0	20.0		
October	16	43.7	56.3		
November	7	71.4	28.6		
December	6	60.0	40.0		
		Season			
Spring	19	63.2	36.8	1.1019	0.797
Summer	15	53.3	46.7		
Autumn	28	57.1	42.9		

Table 2 – contd.

1	2	3	4	5	6
Winter	19	47.4	52.6		
		Abattoir			
I	49	77.6	22.4	24.303	0.000
II	32	21.9	78.1		
		Litter			
Peat	41	70.7	29.3	4.718	0.094
Straw	2	0.0	100.0		
Sawdust	12	75.0	25.0		
		Ventilation system			
Rear wall	2	0.0	100.0	15.575	0.016
Shelter	12	81.5	18.5		
Shelter/Rear	7	71.4	28.6		
Combined	27	0.0	100.0		
Transverse, longitudinal	3	66.7	33.3		
Exhaust	2	0.0	100.0		
Shelter and wall	2	75.0	25.0		
		Windows			
Yes	36	75.0	25.0	1.704	0.192
No	19	57.9	42.1		
		Chlorination of water			
Yes	36	75.0	25.0	1.704	0.192
No	19	57.9	42.1		
		Anteroom			
Yes	52	73.1	26.9	4.933	0.026
No	2	0.0	100.0		
		Boot biosecurity			
Yes	52	73.1	26.9	4.933	0.026
No	2	0.0	100.0		
		Downtime prior to placement date			
≤ 14 days	44	70.5	29.5	0.001	0.977
>14 days	10	70.0	30.0		
		Flock divided			
Yes	41	75.6	24.4	0.632	0.427
No	11	63.6	36.4		
		Age of house			
New	41	75.6	24.4	0.307	0.580
Old	9	66.7	33.3		
		The number of mortality in broiler flock			
≤5%	49	73.5	26.5	0.531	0.466
>5%	2	50.0	50.0		

The analysis of the impact of weather conditions on *Campylobacter* status of the broiler flock showed that the risk of broiler flock contamination with *Campylobacter* increased in July–September and November–January (P=0.031) and peaked in July–August (Table 4). The season did not have an effect on the broiler flock contamination with *Campylobacter* spp. Based on this, presumably differently adapted *Campylobacter* populations may occur at different time periods, and thus the effect of the season may be insignificant.

Table 3. Effect of weather conditions, farm and health at slaughter factors on broiler flocks contamination with *Campylobacter* based on Mann–Whitney Z criteria

	The number of flocks	*Campylobacter*-positive flocks, mean	*Campylobacter*-negative flocks, mean	Z	P
Temperature	81	8.0	8.8	–0.771	0.441
Humidity	81	63.9	63.9	–0.382	0.702
Number of sheds	55	2.0	1.8	–2.469	0.014
Slaughter age	81	40.5	40.6	–0.622	0.534
Average weight per bird at slaughter	81	1.8	2.1	–3.882	0.000

Farm-associated risk factors analysis

The flocks examined in the present study were grown at 16 different broiler farms. To estimate the effect of the farm on the *Campylobacter* status of the broiler flock we calculated the Spearman correlation coefficient. The analysis revealed that broiler flock contamination with *Campylobacter* spp. is farm-dependent (P<0.01). Out of the 16 farms, the highest contamination of broiler flocks (>50%) with *Campylobacter* was found at the farms H, F, C and B (Figure 1). The analysis of farm related risk factors included litter, ventilation system, windows, chlorination of water and age of house (Table 2). Further, the farm related factor analysis showed that farms having the houses with shelter and wall ventilation, shelter and rear ventilation or transverse, longitudinal ventilation system increased the risk (P=0.016) of producing *Campylobacter*-positive broiler flocks (Table 2). An increased risk was also significantly associated with the farms having more than two broiler houses at the same location (Table 3). Lastly, the presence of the anteroom and boot dips also had negative effect (P=0.026) on the *Campylobacter* status of the broiler flock (Table 2). Interestingly, at the farms associated with an increased production of *Campylobacter*-positive flocks three risk factors, such as ventilation system, anteroom and boot biosecurity were identified, whereas the distribution of these risk factors was lower at the farms producing lower numbers of *Campylobacter*-positive flocks (Table S3).

Table S3. Farm significant risk factors analysis by company

	Risk factors		Company															
			A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
Anteroom	Yes	*Campylobacter*-positive	1	22	3	2	0	1	ND	6	ND	ND	ND	0	2	0	1	ND
		Campylobacter-negative	1	5	1	1	2	0	ND	0	ND	ND	ND	1	2	0	1	ND
	No	*Campylobacter*-positive	0	0	0	0	0	0	ND	0	ND	ND	ND	0	0	0	0	ND
		Campylobacter-negative	0	0	0	0	0	0	ND	0	ND	ND	ND	0	0	2	0	ND
																		ND
Boot biosecurity	Yes	*Campylobacter*-positive	1	22	3	2	0	1	ND	6	ND	ND	ND	0	2	0	1	ND
		Campylobacter-negative	1	5	1	1	0	0	ND	0	ND	ND	ND	1	2	2	1	ND
	No	*Campylobacter*-positive	0	0	0	0	0	0	ND	0	ND	ND	ND	0	0	0	0	ND
		Campylobacter-negative	0	0	0	0	2	0	ND	0	ND	ND	ND	0	0	0	0	ND
																		ND
Ventilation system	Shelter	*Campylobacter*-positive	0	22	0	0	0	0	ND	0	ND	ND	0	0	0	0	0	ND
		Campylobacter-negative	0	5	0	0	0	0	ND	0	ND	ND	0	0	0	0	0	ND
	Exhaust	*Campylobacter*-positive	0	0	0	0	0	0	ND	0	ND	ND	0	0	0	0	0	ND
		Campylobacter-negative	0	0	0	0	0	0	ND	0	ND	ND	1	1	0	0	0	ND
	Transverse, longitudinal	*Campylobacter*-positive	0	0	0	0	0	1	ND	0	ND	ND	0	0	0	0	1	ND
		Campylobacter-negative	0	0	0	0	0	0	ND	0	ND	ND	0	0	0	0	1	ND
	Shelter and wall	*Campylobacter*-positive	1	0	0	0	0	0	ND	6	ND	ND	0	0	2	0	0	ND
		Campylobacter-negative	1	0	0	0	0	0	ND	0	ND	ND	0	0	2	0	0	ND
	Shelter and rear	*Campylobacter*-positive	0	0	3	2	0	0	ND	0	ND	ND	0	0	0	0	0	ND
		Campylobacter-negative	0	0	1	1	0	0	ND	0	ND	ND	0	0	0	0	0	ND
	Combined	*Campylobacter*-positive	0	0	0	0	0	0	ND	0	ND	ND	0	0	0	0	0	ND
		Campylobacter-negative	0	0	0	0	2	0	ND	0	ND	ND	0	0	0	0	0	ND
	Rear wall	*Campylobacter*-positive	0	0	0	0	0	0	ND	0	ND	ND	0	0	0	0	0	ND
		Campylobacter-negative	0	0	0	0	0	0	ND	0	ND	ND	0	0	0	2	0	ND

*ND – not determined.

Table 4. Occurrence of various pathologies (averages) in *Campylobacter*-positive and -negative broiler flocks

	Campylobacter-positive flocks		*Campylobacter*-negative flocks		Z	P
	mean	standard deviation	mean	standard deviation		
Ascites	6.8	9.5	17.1	17.7	–3.494	0.000
Skin lesions	0.9	3.8	1.2	4.5	–0.104	0.917
Runts	8.2	5.5	6.0	7.3	–2.541	0.011
Septicaemia	0.3	1.5	0.3	0.9	–1.091	0.275
Hock marks	0.3	1.8	8.3	50.0	–0.177	0.860
Pad burn	551.3	1448.2	1096.7	1799.4	–0.611	0.541
Breast blisters	14.6	96.9	33.3	200.0	–0.759	0.448
Pericarditis	2.2	4.6	1.5	4.5	–1.729	0.084
Perosis	0.0	0.3	0.1	0.3	–0.159	0.874
Green leg	1.8	5.0	6.4	7.4	–4.239	0.000
Subcutaneous pus	0.0	0.1	0.1	0.7	–0.177	0.860
Enlarged liver	0.8	2.3	0.4	0.9	–0.090	0.928
Necrotic foci	7.4	12.9	4.4	6.3	–0.607	0.544
Peritonitis/Perihepatitis	5.9	6.9	1.8	3.4	–2.921	0.003
Other pathology rejections	5.8	16.6	10.7	21.6	–3.680	0.000

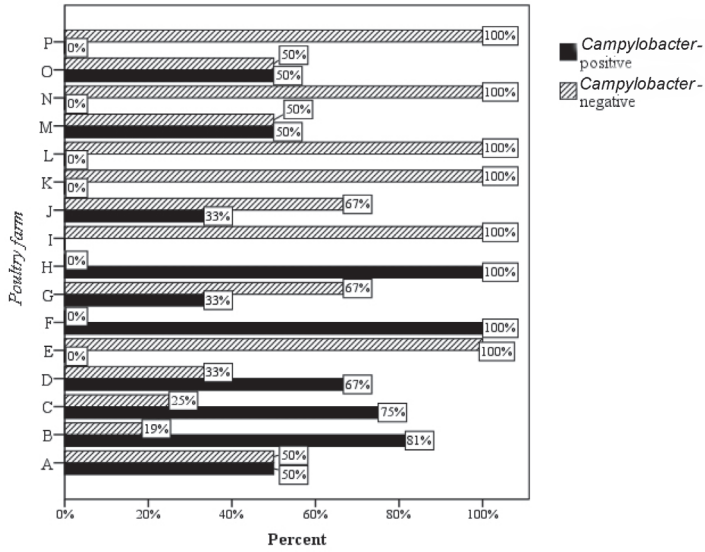

Figure 1. Distribution of *Campylobacter*-positive and *Campylobacter*-negative broiler flocks in association with the poultry farm (r=0.410, P<0.01=0.000)

Broiler flock-associated risk factors analysis

The analysis of flock-associated factors included broiler slaughter age, the percentage of birds found dead at the farm, number of birds arrived at the abattoir and various pathologies (Tables 3 and 4). The results revealed that only the average weight of broilers at slaughter, runts and peritonitis/perihepatitis had the effect on

Campylobacter status of the broiler flocks (Tables 3 and 4). The average weight per bird of *Campylobacter*-positive broiler flocks was significantly lower than the average weight of *Campylobacter*-negative broiler flocks (P=0.000) (Table 3). The data showed that *Campylobacter*-positive broilers flocks had significantly (P<0.05) higher frequencies of runts and peritonitis/perihepatitis (Table 4).

Table 5. Multiple comparison of risk factors associated with broiler flocks contamination with *Campylobacter* spp. by ENTER method

	B	Wald	Exp (B)	P
The effect of weather				
Temperature	0.012	0.181	1.012	0.671
Humidity	–0.005	0.013	0.995	0.910
Farm risk factors				
Number of sheds	–4.445E+01	0.000	4.944E–20	0.999
Litter	–1.096E+01	0.000	0.000	0.999
Ventilation system	–3.574E–01	0.270	0.700	0.603
Windows	22.141	0.000	4.127E+09	0.999
Chlorination of water	–2.226E+01	0.000	2.159E–10	0.999
The age of house	0.257	0.066	1.293	0.797
House risk factors				
Anteroom	22.550	6.296E–07	6.216E+09	0.999
Downtime prior to placement date	0.393	0.249	1.481	0.618
Flock divide	–1.333E–01	0.023	0.875	0.880
Health at slaughter risk factors				
Age at slaughter	–0.495	4.275	0.610	0.039
The number of mortality	0.005	0.132	1.005	0.717
Line count at slaughter	0.000	0.010	1.000	0.921
Average weight per bird at slaughter	5.365	6.921	213.696	0.009
Ascites	0.075	3.400	1.078	0.065
Skin lesions	–0.103	0.418	0.902	0.518
Runts	–0.158	3.006	0.854	0.083
Septicaemia	0.510	3.297	1.665	0.069
Hock marks	0.020	0.104	1.020	0.747
Pad burn	0.000	1.299	1.000	0.254
Breast blisters	0.002	0.348	1.002	0.556
Pericarditis	0.187	2.936	1.206	0.087
Perosis	–1.838	1.021	0.159	0.312
Green leg	0.057	1.074	1.059	0.300
Subcutaneous pus	1.998	1.286	7.377	0.257
Enlarged liver	–0.076	0.046	0.926	0.830
Necrotic foci	–0.014	0.075	0.986	0.784
Peritonitis/perihepatitis	–0.175	2.586	0.840	0.108
Other pathology rejections	–0.027	1.126	0.973	0.289

Multiple comparison

Multiple comparisons using the ENTER method for the risk factors assigned to four groups (Table 5) revealed that there was no single factor having significant ef-

fect on the *Campylobacter* status of the flock in association to weather-associated, farm-associated and flock-associated groups of risk factors. The age at slaughter (P=0.039) and the average weight per bird at slaughter (P=0.009) had statistically significant effect on broiler carcasses contamination with *Campylobacter* spp. (Table 5). This reveals that of all risk factors examined broiler health at slaughter has the highest impact on broiler contamination with *Campylobacter* spp.

Discussion

Poultry are considered to be the main source of human campylobacteriosis. The main risk factor for *Campylobacter* infection in the European Union (EU) is consumption of poultry meat, especially raw or undercooked broiler meat (Wingstrand et al., 2006). Previous studies indicate that cross-contamination of *Campylobacter* during slaughtering is difficult to control (Hue et al., 2010; Ansari-Lari et al., 2011; Allain et al., 2014). Therefore, to reduce human cases of infection, pathogens should be controlled from the primary production stage by preventing colonisation of broilers (EFSA Journal, 2014). The development of the effective control strategies at this stage is dependent on the identification of factors leading to the increased contamination of broiler flocks with *Campylobacter*. In this study we investigated a variety of factors at the farm, at the flock, and at the abattoir levels, as well as the impact of the environmental factors, that may influence the spread of *Campylobacter* at the first stage of broiler meat production.

Campylobacters were recovered from 59.3% flocks sampled at the two abattoirs in Lithuania. Similar or higher broiler flock prevalence (from 54.0% to 79.2%) was also reported in Reunion Island (Henry et al., 2011), in France (Allain et al., 2014), and in the UK (Lawes et al., 2012). In agreement with findings in the UK and France (Lawes et al., 2012; Allain et al., 2014; EFSA Journal, 2014), *C. jejuni* was found as the dominant species in broiler flocks. Due to different sampling procedures, sample size, and isolation protocols used *Campylobacter* prevalence in different studies cannot be directly compared. It was previously proposed that *Campylobacter* prevalence may also be dependent on the climatic conditions of the country. As an example, the weather in the UK is humid and warm during all seasons, which may lead to higher prevalence of *Campylobacter* (Lawes et al., 2012), whereas in cold climate countries, such as Finland and Estonia usually prevalence of *Campylobacter* spp. in broiler flocks is very low (EFSA Journal, 2014). Seasonal variation in *Campylobacter* prevalence in broilers, with a peak in the summer has been previously reported from several countries in northern Europe, e.g. Sweden (Hansson et al., 2007), Norway (Hofshagen and Kruse, 2005), France (Lawes et al., 2012) and Netherlands (Bouwknegt et al., 2004). In agreement to this, we showed that March and the period from July to September was statistically significantly associated with an increased risk of *Campylobacter* contamination in broiler flocks. The peak of 100% of contaminated broiler flocks was observed in July and August. Interestingly, the high proportion of contaminated broiler flocks was also observed in January. According to the published data, *Campylobacter* are sensitive to low temperatures (Hughes et al., 2009; Silva

et al., 2011; Vashin and Stoyanchev, 2011) and, therefore, have lower chances to survive in the environment and spread to the flocks. An increase of *Campylobacter* contaminated meat in January was previously reported in Lithuania, however, it was associated with the increase of samples positive for *C. coli* (Kudirkienė et al., 2010). Contrary to this, *C. coli* and *C. jejuni* proportion remained stable through the whole sampling period in the examined broiler flocks, and thus, may be associated with different survival properties of *C. jejuni* strains as it was observed in other studies (Lawes et al., 2012; Allain et al., 2014). Additionally, we did not observe significant association between the broiler flocks contamination with *Campylobacter* and the season. We speculate that one possible reason for the multiple peaks of *Campylobacter* prevalences is that broilers were sampled at the slaughterhouse but not at the farm, where contamination usually occurs through the environment, especially in summer months with many vectors such as flies involved (Hald et al., 2008). The observed peaks at the slaughterhouse level may be random, and possibly associated with an incomplete disinfection during transportation or low biosecurity in initial processing of broiler batch that may occur when the sales of broilers are increased and procedures are followed improperly.

Statistical data analysis revealed that broiler flock contamination with *Campylobacter* was significantly associated with broiler company where broilers were reared. Out of 16 companies examined in this study, four companies had the highest levels of *Campylobacter*-positive broiler flocks. Six risk factors, such as month, number of sheds, ventilation system, anteroom, boot biosecurity, average weight per bird at slaughter, identified at the farm and flock level, were also present in these farms. Poultry companies have detailed programs for the prevention of the introduction of disease into flocks (BC Poultry Association Biosecurity Committee and Cox, 2006). Small failures in the following biosecurity, biosafety or biocontainment procedures at the farm lead to the increased risk of bacteria spread between the flocks (Newell and Fearnley, 2003; Newell et al., 2014). In line with previous reports (Refregier-Petton et al., 2001; Arsenault et al., 2007; Henry et al., 2011; Chowdhury et al., 2012), individual risk factor analysis showed that contamination of the flock by *Campylobacter* was increased when there were more than two broiler houses in the farm. With every additional house there is an increased number of staff and biosecurity barriers to be passed. Importantly, the first house that becomes positive provides a massive source of cross-contamination for all the remaining houses. *Campylobacters* from the external environment into the houses may be transported through the utilities (litter, feed), by human activities (veterinarians, farmers, staff), or by the entrance of wild animals and birds. Human traffic is a very important vehicle of *Campylobacter* entrance and increases with the number of staff members assigned to the broiler house and the number of visits undertaken per day (Hofshagen and Kruse, 2005; Huneau-Salaun et al., 2007). Farm staff handling livestock, especially broilers, increases the risk of carrying bacteria from their farms (Huneau-Salaun et al., 2007).

Campylobacters can be found on hands, boots and clothes of farm staff, catchers (Ramabu et al., 2004), though the risk of transmission can be reduced if standard farm worker hygiene recommendations are followed at the farm. The use of house specific boots (Bull et al., 2006), clothes (Bouwknegt et al., 2004) and the effective

use of boot dips (McDowell et al., 2008) are all associated with a reduced risk of flock contamination. We found that the presence of boot dips and anteroom at the farm had negative effect on the *Campylobacter* status of the broiler flocks examined in this study. Both of them serve as hygiene barriers that should physically separate the dirty outside environment from the clean and protected inside environment. However, due to improper anteroom and boot dip care or employee negligence they may actually become the main and persistent source of *Campylobacter* cross-contamination between the flocks (Newell et al., 2008).

In addition to risk factors discussed previously, we found that broiler houses with shelter and wall ventilation, shelter and rear ventilation or transverse, longitudinal ventilation systems were associated with *Campylobacter*-positive status of flock. An increased risk of *Campylobacter* colonisation in the houses with vertical, or vertical and horizontal ventilation shafts compared to horizontal vents was reported in several studies (Gibbens et al., 2001; Barrios et al., 2006; Guerin et al., 2007; Vandeplas et al., 2008). Above mentioned studies suggested that vertical fans are a source of heat for wild birds, which may lead to *Campylobacter*-positive bird droppings falling into the house either directly if the fan is not fully bird-proof, or washed in by rain. Such risk explanation is highly possible as many recent studies show that more than 50% of wild birds contain *Campylobacters* in their intestines (Ramonaite et al., 2014).

We also demonstrated that *Campylobacter* status of the broiler flock was associated with the abattoir where it was slaughtered. In particular, higher numbers of *Campylobacter*-positive broilers were slaughtered at abattoir I than in abattoir II. The environment of the slaughterhouse plays major role in *Campylobacter* cross--contamination from positive to negative broiler flocks and is impossible to prevent (Frediani and Stephan, 2003). However, differences between slaughterhouses have also been reported previously (Comin et al., 2014; Messad et al., 2014) and could be related to different hygiene level as well as slaughterhouse equipment differences. We found that broiler flock contamination with *Campylobacter* was higher in flocks with runts and lower average weight per bird at slaughter. Abattoir equipment is designed for a certain weight of birds to be slaughtered. If the weight of a bird is lower or exceeds the requirements, the guts will be damaged and *Campylobacters* will spread to other parts of the carcass and also on the slaughtering equipment, which will become the source of contamination to other carcasses (Posch et al., 2006).

The bird weight is directly associated with bird health. If there are infections, it may lead to the lower weight of bird. This may indicate that broiler carcasses with the reduced weight tend to have higher risk to be damaged and spread *Campylobacter* spp. to the outside. Out of 15 pathologies examined in broiler flocks at the slaughter only runts and peritonitis/perihepatitis were associated with the increased contamination by *Campylobacter* spp. An association between *Campylobacter* flock positivity and bird health has been suggested by several authors (Humphrey, 2006; Bull et al., 2008; Lawes et al., 2012) where among other pathologies, runts and peritonitis/perihepatitis were found to be important risk factors for higher contamination with *Campylobacter* as well. As it was mentioned above, runts increase the risk of cross–contamination during slaughering and therefore they should be culled during the rearing period or rejected at the point of hanging on at the slaughter. The

other pathology peritonitis may be a consequence of the inflammation caused by *Pseudomonas*. Several reports indicate that *Pseudomonas* have positive effect on *Campylobacter* survival in various conditions (Trachoo et al., 2002; Sanders et al., 2007; Hanning et al., 2008; Ica et al., 2012), thus it may also improve *Campylobacter* survival within the host.

In conclusion, *Campylobacter* spp. were isolated from 59.3% of the 81 slaughter batches and with a higher proportion of flocks colonised by *C. jejuni*. *Campylobacter* spp. infection in standard broiler flocks could be linked to several factors of which broiler health associated factors had the highest impact on *Campylobacter* status of the flock. The higher risk of colonisation was also dependent on company where broilers were raised, and on the abattoir where they were slaughtered. Individually, different biosecurity and hygiene measures at the farm, related with the type of ventilation system, number of houses, presence of anteroom and boot dips had impact on broiler flock contamination with *Campylobacter*. The findings reported here provide a robust estimate of *Campylobacter* spp. prevalence and risk factors associated with *Campylobacter* colonisation in Lithuania broiler population and as such can be used as a representative baseline comparison for future monitoring. Hygiene practices and biosecurity measures could reduce *Campylobacter* colonisation at the beginning of the food production chain.

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