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Analysis of farm specific risk factors for *Campylobacter* colonization of broilers in six European countries

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ABSTRACT

This study presents on-farm risk factors for the colonization of broiler flocks with *Campylobacter* based on comparable data from six European countries: Denmark, the Netherlands, Norway, Poland, Spain, and the UK. The study includes explanatory variables from a large questionnaire concerning production, farm management procedures and farm conditions, climate data on mean temperature, sunshine hours, and precipitation, as well as data on *Campylobacter* status of broiler flocks. All together the study comprises data from more than 6000 flocks. The data were analysed using a generalized linear model. Due to a large number of parameters, some collinearity and relatively many missing values, the model was analysed by a method using all available cases at each step in the modelling process. The modelling process includes backwards elimination and forward selection. Several approaches were furthermore explored by applying different strategies for categorizing explanatory variables and for selecting and eliminating variables in the model.

Despite national differences in broiler production, common risk factors for *Campylobacter* colonization of broiler flocks were identified across all six countries. These were generally related to inadequate biosecurity. Identified risk factors were: broiler houses older than 15 years, absence of anterooms and barriers in each house, shared tools between houses, long downtime, and drinker systems with bells or cups. Also, the risk of broiler flocks becoming colonized with *Campylobacter* was clearly affected by country. In descending order, broiler flocks were more likely to be colonized in Poland, the UK, Spain, the Netherlands, Denmark and Norway due to country specific factors that could not be explained by the identified risk factors or any other variables from the questionnaire. The seasonality observed for prevalence values was described by the monthly mean temperature reported in the study, i.e. the higher the temperature, the higher the prevalence of positive flocks.

1. Introduction

Many attempts have been made to understand the epidemiology of *Campylobacter* in broilers in search of effective control measures for preventing *Campylobacter* colonization of indoor commercial broiler flocks. *Campylobacter* can be transferred to humans e.g. via broiler meat or via the environment being contaminated by feces from production animals such as for example broilers and cattle and thereby cause campylobacteriosis, which is the most common cause of foodborne gastrointestinal illness in the EU and the rest of the industrialized world (EFSA and ECDC, 2015; WHO, 2012). Studies have shown that the main route of introduction of *Campylobacter* into broiler flocks is from

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the environment outside the broiler house due to one or more breaches in biosecurity (Adkin et al., 2006; Hald et al., 2000; Lyngstad et al., 2008). Hence, controlling *Campylobacter* in housed birds is primarily a question of strict management practices with a high level of biosecurity and broiler houses that are closed off to the environment. There are, however, many factors involved in achieving a high level of biosecurity and some are more important than others. The most important risk factors are those, where the risk of carrying *Campylobacter* into the broiler houses is greatest, for example by contaminated air, shoes, clothes, flies, etc.

Previous work from Northern Europe has shown that housed flocks are significantly more likely to be *Campylobacter*-positive during the summer period (Jonsson et al., 2012; Jore et al., 2010), when ambient temperatures are high compared with the winter period. This is partly related to increased airborne transmission of *Campylobacter* due to high airflows into the houses during warm periods (Hald et al., 2008). The bacteria may be present in dust or, more importantly, in insects (Bahrndorff et al., 2013; Hald et al., 2007). Climatic factors may, therefore, partly explain the higher *Campylobacter* prevalence in flocks in parts of Eastern and Southern EU (EFSA, 2010), where ambient temperatures are higher and where there is a constant requirement for higher airflow through the houses. Given variations in climate and broiler flock prevalence across Europe, we speculated if risk factors for flock colonization were similar or different in Northern, Eastern and Southern Europe. To our knowledge, this has not previously been investigated in one study.

Comprehension and awareness of country specific risk factors can guide the choice of the preventive measures in a country to ensure optimal effect, and thus lead to safer broiler meat production within the country. Identification of common risk factors that are applicable in all EU countries is also vital, given the EU-wide nature of broiler production and marketing and the high incidence of campylobacteriosis across the EU.

The objective of this study was therefore to study on-farm risk factors for the colonization of broiler flocks with *Campylobacter* based on comparable data from six European countries. Previously, we analyzed risk factors in two Northern European countries (Denmark and Norway) (Høg et al., 2016). In the present study, we expand the risk factor study to also include data from the Netherlands, Poland, Spain and the UK, and we included climate related variables to the list of risk factors. The study was designed to investigate risk factors related to climate, geography and on-farm management practices in housed broiler flocks (the most common type of broiler production in EU (EFSA, 2011). The analysis was based on a method that uses all available cases at each step in the modelling process (backwards elimination and forward selection) (Sommer et al., 2013). For every fifth step of backwards elimination a forward selection was run to include earlier eliminated variables if *p*-values were less than 0.10.

2. Materials and methods

2.1. Questionnaire, Campylobacter status and climate data

The farm specific variables were obtained through a standardized questionnaire including 43 questions concerning production, farm management procedures, farm conditions, etc. Further information on the questionnaire can be found in Høg et al. (2011), where the questionnaire is also presented in full. Some questions were excluded due to difficulties in interpretations or too little variation in the responses.

In Denmark and Norway, *Campylobacter* data for flocks on farms that responded to the questionnaire were obtained through national surveillance programmes (Høg et al., 2016). In Norway, only flocks slaughtered from May to October in 2010 and 2011 were tested for *Campylobacter,* in total 1400 flocks. In Denmark, full annual datasets were obtained from 2010 and 2011, in total 3864 flocks. *Campylobacter* flock data from Poland, Spain and the UK were obtained through a two-year longitudinal study within the period 2011–2013, where all flocks in the study houses were tested for *Campylobacter*. Furthermore, *Campylobacter* results were obtained from 276 flocks from Poland, 201 flocks from Spain and 219 flocks from the UK. In the Netherlands, *Campylobacter* status was collected from 221 flocks during 2012 and 2013. All flocks were tested prior to or at the time of catching the first batch of birds from the flock (first thin). All flocks from Denmark, Norway and the Netherlands were sampled on farm by boot swabs and tested using PCR, as described by Lund et al., (2004). In Poland, Spain and the UK, caeca from 10 birds per flock were sampled at the slaughterhouse and pooled before microbial analysis; isolation and confirmation of *Campylobacter* organisms in caecal contents were undertaken as described in ISO 10272-1:2006. At least one *Campylobacter* isolate per batch was speciated using phenotypic methods as described in ISO 10272-1:2006 or by PCR as described by Klena et al., (2004).

Climate data were collected from weather stations as close as possible to the farms included in the study. This meant that two-three climate datasets were obtained from Norway, Poland, Spain and the UK. However, due to small country size for Denmark and the Netherlands, only one data set per country was included for these two countries. Climate data were matched to each flock by the month of slaughter. Three climate variables were chosen to represent the climate; monthly outside mean temperature, monthly total precipitation, and monthly total sunshine hours. These three variables were chosen, because a preliminary analysis of the Danish data had shown their ability to describe the seasonality in the broiler flocks prevalence (data not shown).

2.2. Data preparation

Questionnaire data were prepared for analysis to improve data quality and to maximize the number of data and variables in the final model. This step was essential for running the model with as many variables as possible. Wherever reasonable, missing values were filled in, and the number of parameters was reduced by merging variables or categories. Furthermore, highly correlated variables were excluded.

2.2.1. Merging

To reduce the number of parameters, some of the categories within variables were merged, especially for variables with a large number of categories and many combinations of these. Variables were merged based on expert's opinions and the empirical prevalence estimates. If experts suggested categories to be merged, but the empirical prevalence estimates were largely different, then the categories were not merged. Two questions had a hierarchical structure and were merged in order to avoid collinearity, e.g. the merging of variables concerning downtime between flocks (*Using downtime (yes/no)* and *Duration of downtime)*. If no downtime, the duration of downtime was set to zero and, thus, became part of the variable *Duration of downtime* (from now on referred to as *Downtime)*.

2.2.2. Collinearity

Correlation between two or more variables (multicollinearity) may cause problems running the model as a consequence of an unsuccessful approximation to the inversed Hessian matrix (Altman et al., 2004). In this study, we removed one of the highly correlated sets of variables from the model initially (to make the model run) and later allowed the removed variables to re-enter the model in the forward selection.

Table 1
Percentage (%) of missing values per country and in all countries.

Variables	DK	ES	NL	NO %	PL	UK	All countries
Rodent control frequency	11	80	16	23	28	5	21
House surroundings, non-access areas	3	0	16	27	86	45	20
Time of thinning (to remove the birds)	72	0	5	89	14	30	68
Time between thinning	72	5	5	90	10	45	69
Cats access to broiler house	36	90	53	49	76	50	48
Dogs access to broiler house	35	73	35	45	49	27	42

2.2.3. Missing values

Missing values in the explanatory variables are problematic in statistical analyses. They may obstruct running a model with relative many parameters and affect the conclusions if they occur systematically, which may reduce the representativeness. Furthermore, they imply a loss of information, which often results in less accurate estimates of the parameters. Therefore, missing values were filled out by using information from farmers' remarks in the questionnaire whenever possible and they were tested for systematic missingness. More than 95% of the data records contained one or more missing values. All variables with 20% or more missing values were examined for systematic missingness. This was tested according to the variable Country by computing the relative numbers of missing values for each country and applying a chi-square test (Fishers exact test in cases of few observations). Due to multiple chi-square tests, the significant level was adjusted by using the Bonferroni correction (Bonferroni, 1936).

Further on, a few questionnaire variables were excluded due to difficulties in interpreting the answers and some variables had too little variation to be included in the model (Høg et al., 2016).

2.3. Data analyses

2.3.1. Statistical analysis

The final dataset, which comprised more than 40 variables (climate plus questionnaire variables) from six countries, was analyzed by a multivariable variance analysis. The prevalence, $p_{i,j,k,\ldots}$ was the response variable in the model and was defined as the proportion of *Campylobacter* positive flocks out of the total number of broiler flocks produced a given month on a given broiler farm. Since the response variable was binomially distributed, a generalized linear model was applied and the response variable was transformed using a logit link function:

$$logit\left(p_{i,j,k,\ldots}\right) = \log\left(\frac{p_{i,j,k,\ldots}}{1-p_{i,j,k,\ldots}}\right) = \beta_0 + \beta_1 X_i + \ldots + \beta_{4,k} \qquad (1)$$

where, $p_{i,j,k,\ldots}$ is the prevalence value, $\beta_1 X_i$ express a regression term with temperature, $\beta_{4,k}$ express a categorical term. The number index for the betas (1, 2, …) refers to the question number given in Table 1 in Høg et al. (2016).

The analyses were carried out using PROC GENMOD in SAS (version 9.4, SAS Institute Inc.). This routine requires complete-cases data. Since the study consisted of repeated measurements from the same farms (several records from different months), the model was explored for different variance structures and an overdispersion parameter was found suitable.

The next step was to find out which of the groups within a variable that contributed to an increased risk. Predicted population margins were calculated as the means for a balanced design using the LSMEANS statement in SAS.

$$LSMEANS = logit\left(pp\right) = \sum_{i=1}^{N} logit\left(p_i\right) \cdot 1/N \qquad (2)$$

where p_i is the predicted prevalence estimate in a cell (given by the combinations of parameters for the categorical variables), *pp* is the marginal population estimate, and *N* is the total number of cells. The equally weighting, when calculating the marginal population means, results in marginal population means that are adjusted for influence of other variables in the model. The regressor variable (temperature) was included in the LSmeans estimations as an overall mean temperature, which balanced out the effect of the temperature between the countries. The adjustment of LSmeans made a direct visual comparison of the effect of the parameters possible. In order to test the differences between the parameter estimates, Tukey's multiple comparison tests were applied.

2.3.2. Modelling process

Fig. 1 provides an overview of the modelling process. Before running the actual risk factor analyses, pre-modelling was carried out to determine constants and setup important for modelling seasonality of the *Campylobacter* prevalence. The risk factor analyses started with an initial model including as many main effects as possible but no interaction terms. The model was then reduced using backward elimination and a forward selection routine as described by Sommer et al. (2013) and reached a reduced model with significant variables. Several approaches for the models were then explored, using different strategies for categorizing the explanatory variables. The reduced model, which comprised the largest possible dataset, was selected as the main model and is referred to as such. Finally, the constants (temperature cutoff and shift) in the pre-model were optimized including the newly identified significant variables and the main model was run again.

A previously published model approach was used (Sommer et al., 2013). In brief, the process of identifying significant variables began with an initial model and stepwise the number of variables was reduced. For every fifth step of backwards elimination a forward selection was run to include previously eliminated variables if *p*-values were less than 0.10. This procedure allowed the sample base to expand each time a variable with missing values was removed from the model. Insignificant variables were removed using the following principle; the three variables with the highest *p*-values were selected and among these the one with the largest number of missing values was removed.

Variables that were not in the model could enter the model in the forward selections routines if *p*-values were < 0.1. Interaction terms were included in the model when the number of variables was reduced considerably and thus allowed for inclusion of more variables.

2.3.3. Modelling seasonality

The seasonal distribution of *Campylobacter* prevalence in the broiler flocks, i.e. a higher prevalence during the warmer months and a lower during the colder months of the year, was included in the risk factor analysis. Preferably, the seasonality should be explained by variables in the model instead of a sinus-cosine function, which has been used in other studies (Boysen et al., 2011, Chowdhury et al., 2012). The only variables that varied over the

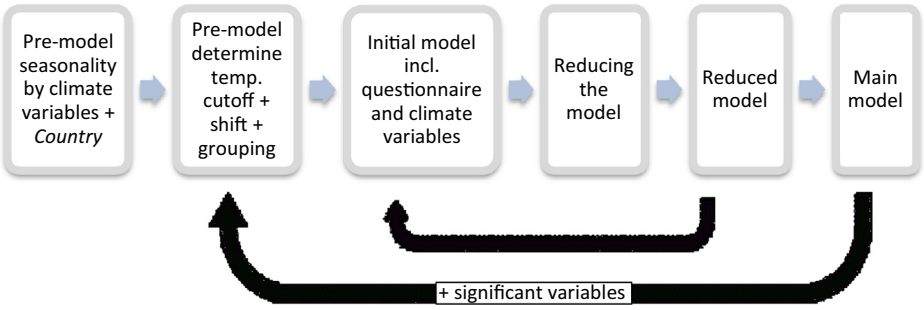

Fig. 1. Modelling flow of the risk factor analyses.

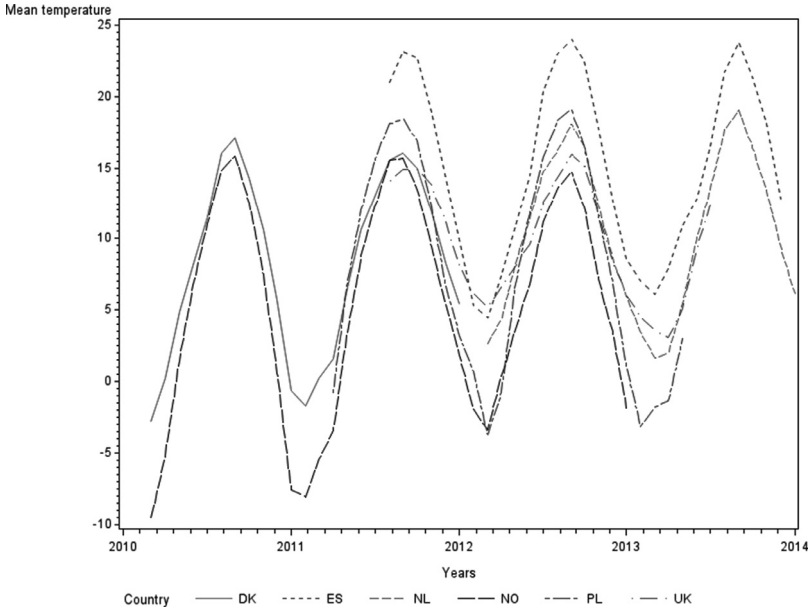

Fig. 2. Seasonality in monthly mean temperatures for six European countries. The temperatures on the y-axis are given as degree Celcius.

months were the climate variables: *Temperature* (mean temperature for a given month), *Sunshine* (total sunshine hours for a given month), and *Precipitation* (total amount of precipitation for a given month). It was therefore explored if these could replace the sinus-cosines functions by running different pre- models. Pre-models only containing *Country* + climate variables were tested. In order to compare the results of the pre-models, the analyses were carried out using the same set of complete data for all pre-models. In Fig. 2, the seasonality in the temperature data from the six countries are shown.

It was examined whether a cutoff for the temperature was necessary, i.e. a value below which the temperature would not affect the broiler flock prevalence significantly. We expected that the *Campylobacter* prevalence to increase with increasing temperature. Since the climate data were reported for the month of which the chickens were slaughtered and not for the period they were grown, the *Temperature* was shifted back in time.

$$Temp_i = Temp_i^* + \left(Temp_{i-1}^* - Temp_i^*\right) \cdot' shift' \quad (3)$$

where $Temp_i$ is the temperature variable after the time shift. $Temp_i^*$ is the original temperature variable at month i and 'shift' is the number of month shifted back in time. The regressor variable *Temp* was split into two variables, by the interaction term *Temp*∗*EU*. The *EU* variable divided the countries into two groups: (1) Denmark and Norway and (2) the Netherlands, Poland, Spain and the UK. This was necessary since the effect of the regressor variable varied between the two groups, i.e. the temperature had a larger effect on the annual prevalence estimates for Denmark and Norway than for the other countries.

Since the variables in the model may have some effect on the estimate of the cutoff and shift (and vice versa) the model was re-run (the loop back in Fig. 1) after having identified the (first) main model. The cutoff temperature and the magnitude of the time shift were optimized by running the main model again now including different combinations of cutoffs and shifts. The combination that resulted in the smallest Akaike information criterion (AIC) (goodness of fit) value for the model was selected.

3. Results

3.1. Pre-analyses

Two pairs of variables were highly correlated: *Age of newest house* and *Age of oldest house*, and *Number of houses* and *No. of birds slaughtered per year* by 0.68 and 0.53, respectively. One from each pair (*Age of newest house* and *Size of production)* were left out from the initial model to enable the model to run.

Six variables had systematically missing values (Table 1). Some of these had reasonable explanations; e.g. for the variables *Time of thinning* and *Time between thinning* Norway had the largest number of missing values, because a small percentage of the Norwegian farms use thinning. Spain had a large number of missing values for the question *Cats access to broiler house,* because very few farms reported having cats on the farm. These six variables were not part of the model initially, but were included at a later stage by the forward selection routine if *p*-values were below 0.1.

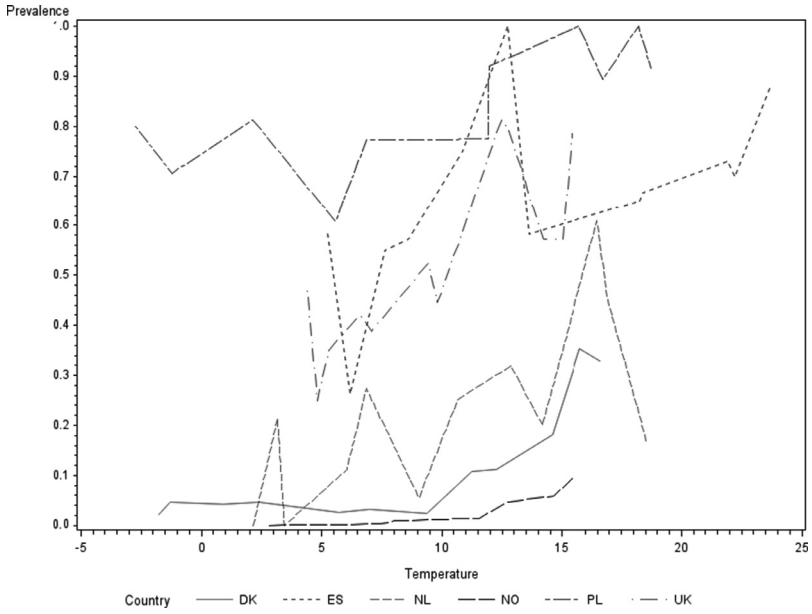

Fig. 3. Mean monthly temperature per country plotted against empirical *Campylobacter* prevalence values. The temperatures are given in degrees Celcius.

Table 2
Pre-models to examine the fit of seasonality by using climate data. Shift, cutoff, and group are all related to the variable *Temperature*. AIC is the Akaike's Information Criterion. The smaller AIC the better model fit.

Pre-models	AIC values
i.1 *Country*, sin, cosines	2669.3
i.2 *Country, Temperature, Sunshine, Precipitation*	2673.5
i.3 *Country, Temperature*+cutoff, *Sunshine, Precipitation*	2672.2
i.4 *Country, Temperature*+shift+cutoff, *Sunshine, Precipitation*	2644.9
i.5 *Country, Temperature*+shift+cutoff+group, *Sunshine, Precipitation*	2610.0

The climate variables described the seasonality better than the sinus-cosine function, i.e. the AIC values for the climate-models i.4 and i.5 were lower than the AIC value for the sinus-cosine model (i.1) (Table 2). In model i.4 *Sunshine* was not significant and in model i.5 *Sunshine* and *Precipitation* were not significant. In other words, *Temperature* in itself seemed to be able to describe the seasonality in the prevalences. Note that shift is moving the temperature back in time; cutoff defines a temperature for which the variable *Temperature* does not have any effect if lower than this cutoff value. In Fig. 3, the *Campylobacter* prevalence = N_positive / N_total per month is plotted against the monthly mean temperature in each of the six countries. In general, the Danish and Norwegian data were used to define an initial cutoff temperature. The variations in data for the other four EU countries were too large to identify a cutoff. 'Groups' were created since Denmark and Norway responded more strongly on the variable *Temperature* compared to the rest of the six countries – the 'group' indicates an interaction between country groups and *Temperature*.

Based on the results in Fig. 3 the temperature cutoff was initially set to 10°C. The models i.3–i.5 were run using this cutoff value (Table 2). The magnitude of the shift was determined by running the model with the variables *Country* + climate variables for different magnitudes of the shift. The fits were compared by using the AIC values to determine the best initial estimate of the shift which was found to be 0.5 month.

3.2. *Campylobacter* prevalence

The observed prevalence values of *Campylobacter* in broiler flocks from the participating farms in the six countries are shown in Fig. 4. No farms from the study were consistently negative throughout the study period. Almost a third of the participating farms in Poland were tested consistently positive throughout the study period.

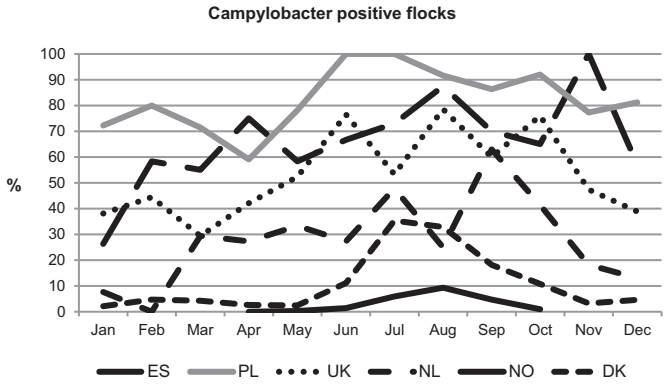

Fig. 4. Observed prevalence of *Campylobacter* in broiler flocks from participating farms in six European countries. The monthly estimates of the prevalence are calculated from several years.

A clear seasonal trend was observed for Danish and Norwegian data with a higher prevalence in the warmer months; June-October (Fig. 4). Also for the Dutch data, there was a clear seasonal distribution, with less than 20% positive flocks from November to February. The seasonal variation was less pronounced in Poland, Spain and the United Kingdom, where the percentage of positive flocks was never below 59%, 26% and 29%, respectively.

Differences in the temperature patterns in each country were considered an important explanatory variable to describe the differences in prevalence values between the countries. Therefore, the mean temperature for the data in the study (temperatures above the cutoff value) for each country were calculated and depictured against the prevalence values. However, there was no unique association between mean temperature in the individual countries and the prevalence (Fig. 5). Especially Poland and the UK had higher prevalence values than expected if the mean temperature was the most important risk factor. Hence, the temperatures explained the seasonality well but could not completely explain the prevalence differences between the countries.

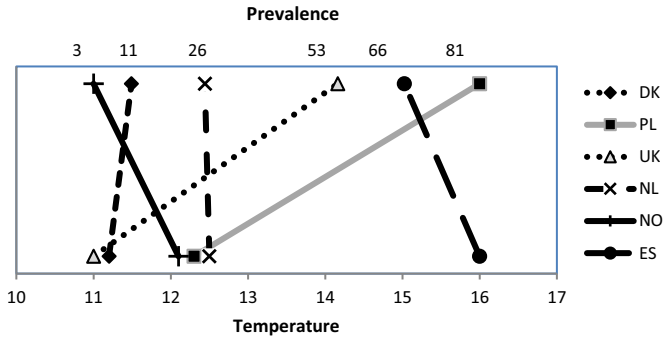

Fig. 5. Relation between mean temperature and mean *Campylobacter* prevalence. The mean temperature was calculated from the study data temperature above the cutoff value of 8°C. Data for Norway only included the summer period (May–October 2012, 2013).

3.3. Data and variables

The initial models included 34 variables and contained 67% of the full data set. This was the largest number of variables for which the models could run. Some variables had a low *p*-value < 0.10 throughout the analyses, while others had low *p*-values only towards the end of the model reduction process, yet others went (out and re-entered) into the model by the forward selection routine. Six variables were permanently excluded from the model due to too little variations in the variable, difficulties in interpretation, or inclusion in other variables. Furthermore, variables with 20% or more missing values were left out from the initial model (*Rodent control frequency, Thinning time, Time between thinning, Reverse ventilation, Dogs access to house, Cats access to house*), as well as two highly correlated variables *Age of newest house* (correlated with *Age of oldest house)* and *No. of birds slaughtered per year* (correlated with *No. of broiler houses*). Having identified the first main model, the model was re-run in order to optimize the cutoff and shift estimates. In Table 3 the different combinations of cutoffs and shifts together with the AIC values are shown. The combination of cutoff and shifts that had the smallest AIC value was a cutoff of 8°C and a shift of 15 days. The model was re-run again from the beginning with the new values of cutoff and shift. The second main model resulted in the same variables as the first and the estimated *p*-values were almost the same as in the first main model (only a few changes on the fourth decimal). This was the final model.

3.4. Results for the main model

The final model was based on 95% of the full dataset and 11 significant variables. These are shown in Table 4 along with their *p*-values, estimates, and the standard errors (SE). Some farms delivered more than one flock in a given month, which explains that the total number of flocks is greater than the total number of data/record lines in the final model.

The mean temperature described the seasonality very well. Shifting the mean temperature 15 days back and using a cutoff of the temperature of 8°C displayed the best fit of the data. Moreover, nine factors were found to have a significant effect on the risk of flocks becoming colonized with *Campylobacter: Country, Age of house, Anteroom+barrier, Downtime, Drinkers, Tools, Age of house∗Country, Age of house∗Anteroom+barrier, Age of house∗Tools*. The interaction term *Age of house∗Country* indicates that the effect of *Age of house* depends on the country. Furthermore, the last two interaction terms showed that the effect of *Anteroom+barrier* and *Tools* depend on *Age of house*.

Table 3
Optimization of temperature cutoff and temperature shift. The main model (on the second loop) was modelled with different values of cutoff and shift. AIC is the Akaike's Information Criterion. The smaller AIC the better model fit.

AIC-values		Temperature cutoff 7°C	8°C	9°C	10°C
Temperature shift	−12 days	2988.2	2986.9	2992.6	3006.7
	−15 days	2985.7	2985.3	2989.9	3003.8
	−18 days	2989.8	2989.5	2994.2	3007.3

In Fig. 6, the marginal population means (LSmeans estimates) are shown together with the 95% confidence intervals for all the variables in the final model. On the y-axes the logit of the LSmean values are given – the higher the values the higher the prevalence of *Campylobacter*. The LSmean values should not be interpreted directly, since it is a value on the logit scale and since it is calculated for a balanced design (unlike our study). The LSmean values only serve to compare the effect estimates for the levels within a variable. For the variable *Country* (Fig. 6h), Denmark and Norway had significantly lower values and Poland significantly higher value than the other countries. The UK, the Netherlands and Spain were not significantly different from each other (tested by the Tukey multiple comparison test).

In general, farms where the newest broiler houses were less than five years old (*Age of house*) had a significantly lower risk of becoming infected by *Campylobacter* than those with older houses (Fig. 6a). There were no significant differences between the two last categories (years 6–15 and > 15). However, when taking a more detailed look at the effect per country, a different trend was observed for Poland, where flocks on the farms with the oldest houses had a lower risk of becoming colonized with *Campylobacter* (Fig. 6i).

Having both anteroom and barrier (*Anteroom+barrier*) as well as designated tools (*Tools*) in all the houses on the farm generally led to significantly lower risk than if these biosecurity measures were not in place in all houses (Fig. 6c and 6d). Designated tools implies that all tools e.g. brooms and shovels, were exclusively used in one broiler house, and not transferred from one house to another. However, when more information was provided, it became clear that the effects of *Anteroom+barrier* and *Tools* were related to the age of the house, and actually had no effect in the oldest houses (Fig. 6f and 6g). *Downtime* was significant; the longer the downtime the higher the risk of flocks becoming colonized (Fig. 6e). Downtime < 10 days had a significantly lower risk than category downtime > 10 days. Also the type of drinker systems appeared to affect the risk, i.e. drinker nipples without cups were associated with the lowest risk (Fig. 6b). The risk estimates for drinker nipples with cups or bells were not significantly different from each other.

3.5. Nearly significant variables

Some variables were close to entering the final model. *Rodent control frequency* was in fact significant, but was left out due to many missing values and systematic missingness. Carrying out rodent control less than four times a year increased the risk. *Water supply* was very close to being significant with a municipal water source as the best, followed by private bore holes and surface water. The *Fly screen* variable was just significant ($p = 0.048$); having screened the house as the best, but since only three Danish farms had fly screens, it was left out from the final model. *Reverse fans* was also significant, but with 35% missing values it was also left out.

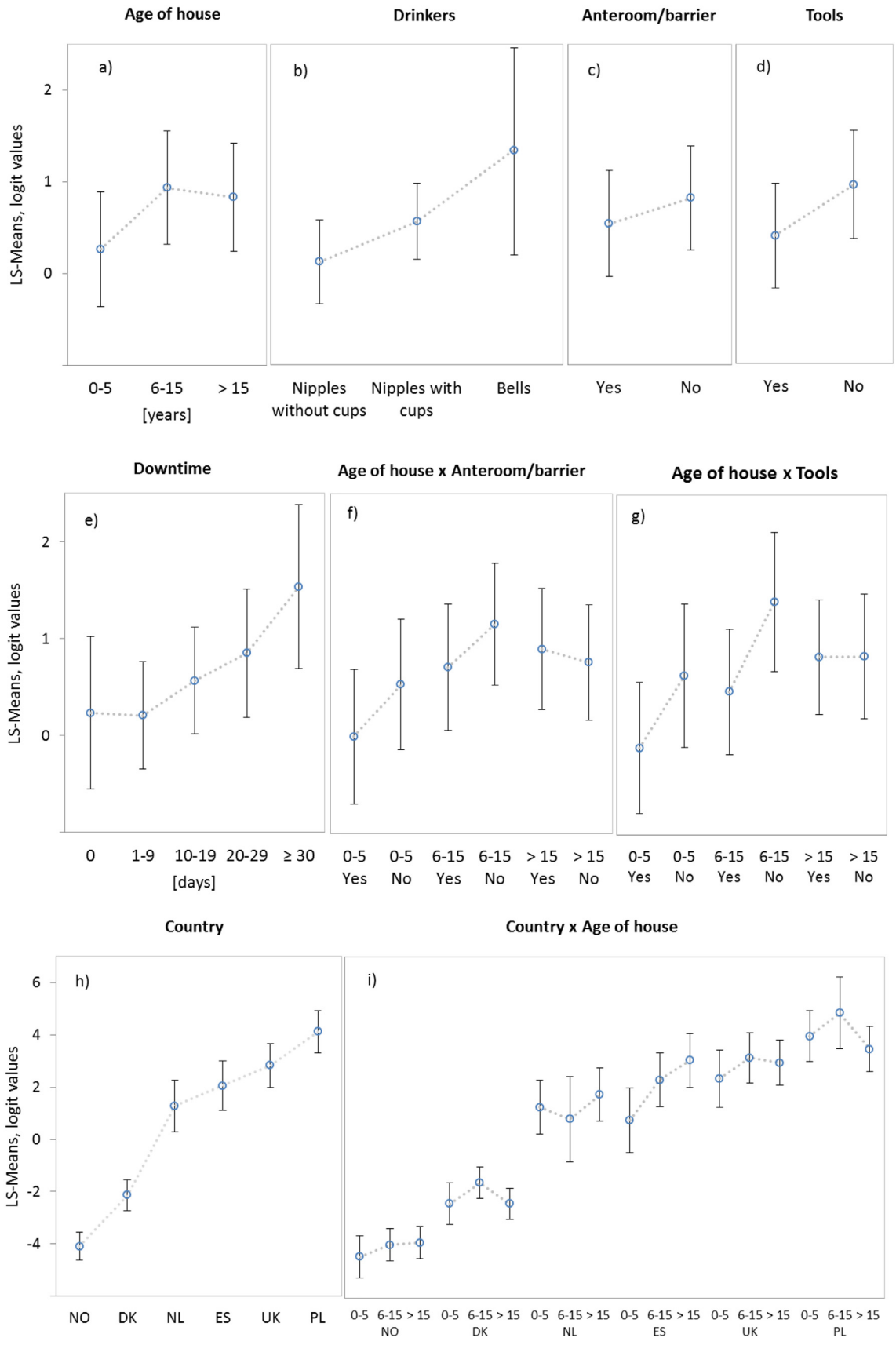

Fig. 6. Marginal population means of the logit prevalence of *Campylobacter* (LSmeans estimates) with the 95% confidence intervals for parameters in the main model. *Age of house* in a) is the age of the broiler house, *Drinkers* in b) refers to the drinking system used in the broiler house, *Anteroom+barrier* in c) refers to having anteroom plus barriers in the broiler house, *Tools* in d) are designated tools for each broiler house, *Downtime* in e) refers to the downtime between crops in the broiler house, f), g), and i) are interaction terms.

Table 4
Significant variables in the final model of risk factors for broilers becoming *Campylobacter* positive in six European countries.

Parameter	Variables	*p*-value	Category		Estimate	SE
β_0	Intercept	–			0.67	0.80
β_1	*Temp* *	< 0.001			0.15	0.03
β_2	*Temp x EU* *	< 0.001	Denmark, Norway		0.21	0.03
			Spain, the Netherlands, Poland, UK		0	0
β_3	*Country* *	< 0.001	Denmark		–5.38	0.48
			Spain		0.08	0.42
			the Netherlands		–1.22	0.40
			Norge		–6.85	0.54
			Poland		0.51	0.37
			UK		0	0
β_4	*Age of house* *	0.002	<5 years		0.1	0.52
			6–15 years		0.94	0.41
			>15 years		0	0
β_5	*Anteroom+barrier*	0.022	Yes		0.14	0.17
			No		0	0
β_6	*Tools* **	0.001	Yes		-0.01	0.19
			No		0	0
β_7	*Downtime* *	0.006	0 days		–1.3	0.46
			1–9 days		–1.33	0.37
			10–19 days		–0.97	0.34
			20–29 days		–0.69	0.35
			> 29 days		0	0
β_8	*Drinkers*	0.001	Nippels without cups		-1.22	0.61
			Nippels with cups		-0.78	0.61
			Bells		0	0
β_9	*Age of house x Country*	0.001	Denmark	0–5 years	0.62	0.55
				6–15 years	0.62	0.45
				>15 years	0	0
			Spain	0–5 years	–1.67	0.70
				6–15 years	–0.92	0.60
				>15 years	0	0
			the Netherlands	0–5 years	0.13	0.63
				6–15 years	–1.12	0.87
				>15 years	0	0
			Norway	0–5 years	0.07	0.60
				6–15 years	–0.27	0.50
				>15 years	0	0
			Poland	0–5 years	1.11	0.62
				6–15 years	1.2	0.81
				>15 years	0	0
			UK	0–5 years	0	0
				6–15 years	0	0
				>15 years	0	0
β_{10}	*Age of house x Anteroom+barrier*	0.019	Yes	0–5 years	–0.67	0.32
				6–15 years	–0.58	0.24
				>15 years	0	0
			No	0–5 years	0	0
				6–15 years	0	0
				>15 years	0	0
β_{11}	*Age of house x Tools*	0.013	Yes	0–5 years	–0.74	0.38
				6–15 years	–0.92	0.35
				>15 years	0	0
			No	0–5 years	0	0
				6–15 years	0	0
				>15 years	0	0
No. of obs. (record lines)	3.986					
No. of flocks	6.042					

Note: The significance values (type 3 *p*-values) and estimates are also given. * denotes that the *p*-values were < 0.10 all the way through the modelling process. ** denotes that the variable (re-)entered the model in a forward selection step. The categories with the estimated value 'zero', are reference categories.

4. Discussion

4.1. Method used

Analyzing data from large surveys can be challenging due to a large number of explanatory variables, and often also a large number of incomplete data records. Data records are said to be incomplete if there is missing values for any of the explanatory variables in the model. Most multivariable statistical routines require complete-cases. Thus, when the number of variables is large, the loss in efficiency can be particularly large if the dataset contains missing values (Little and Rubin, 2002). However, if only the dataset of complete cases are included in the analysis, there is loss of statistical power of the tests due to the decrease in effective sample size. Little and Rubin (2002) presented an available-case method, based on the univariate analyses and includes all available cases, where the variable of interest is present. The method involves estimation of all pairwise covariances between parameter estimates. The advantage of this method is that it makes use of all the available data. The disadvantage is that when variables are

highly correlated, this is not accounted for in the model (Pigott, 2001). The complete-case method is superior to the available-case method, when the correlation is high. Neither method, however, is overall satisfactory. In the present study, we applied a method based on all available cases at each step in the modelling process (backwards elimination+forward selection) (Sommer et al., 2013).

The preparation of the dataset was an essential step, because it allowed us to include more information in the analyses, than if data had not been prepared. Had we not prepared the data and only used complete cases in the model, as little as 4.5% of the dataset would have been included in the model. However, after preparing the data we were able to use 67% of the dataset, and by applying the backward elimination and forward selection as described in the present study, we were able to use 95% of the data in the final model. Many risk factor analyses use only complete-cases observations throughout the modelling process missing out information in the dataset (Arsenault et al., 2006). The idea behind the forward selection routines was to avoid exclusion of variables in the early steps simply due to a relatively small sample base. These variables were continuously tested for inclusion in the model in later steps by the forward selection procedure.

The collinearity problem could have been solved by implementing ridge regression. However, we chose not to use this method since it introduces bias in the estimates, and since collinearity does not reduce the predictive power or the reliability of the model (Voss, 2004; Belsley, 1991). The collinearity may, however, prevent the model from running, but we solved that as described earlier by reducing the number of missing values and merging categories.

For models with no interaction terms and no continuously variables it is not necessary to estimate the LSmean values in order to compare the effect from the different categories of the risk factors. In such cases, the effects can be read directly from the parameter estimates. However, for models including interaction terms there is an advantage in estimating the LSmean values in order to compare the levels (Cai, 2014).

4.2. Significant variables

Two climate related variables (temperature shift and temperature cutoff) were found to have the optimal values of 15 days and 8°C, respectively. In comparison, Patrick and co-workers (2004) found a shift (delay) of 3 weeks to be the optimal value, when mean temperature was used to describe the seasonality in the prevalence values for *Campylobacter* in broiler flock in Denmark. At mean temperatures above 8°C, they reported a large increase in broiler flock prevalence with increasing temperature. Other studies have defined cutoff values of 6°C in Norway (Jonsson et al., 2012).

Most of the risk factors identified in the final model seem reasonable with plausible explanations: *Campylobacter* prevalence in broiler flocks increases with increasing *Age of house* (old houses become less 'biosecure'), absence of *Anteroom+barrier* (no effective biosecurity at the entrance of the houses), absence of designated *Tools* (easy cross contamination between broiler houses), and *Drinkers* with cups (providing a water reservoir where bacteria may grow). Several of these risk factors have also been identified in other studier (Adkin et al., 2006), however in this study, as something new, we have also proven that designated tools and anteroom+barriers only have an effect in new/newer houses and no effect in old houses.

However, a few variables need further explanation. *Downtime* is one such variable. In this study, as reported by Høg et al. (2016), we found increasing prevalence with increasing downtime. Other studies have found that shorter downtimes (less than 9–14 days) are associated with increased risk (Berndtson et al., 1996; Hald et al., 2000; Lyngstad et al., 2008). However, the ratio of farmers that reported not cleaning the house between flocks increased from 0% to 24% as the length of the downtime increased. Moreover, the frequency of poor rodent control (1–3 times a year) increased from 0% to 9% as the downtime period increased. Furthermore, in Spain, the farms that did not perform as well (possibly with a higher risk of having *Campylobacter*) as other farms, were less often selected to produce broilers when the demand for broilers was low. For Spain, this partly explains the association between a long downtime and a high prevalence.

Country is another variable that needs explanation. The final model consists of several significant variables that describe the variation in the data. If the variable *Country* was not significant, the variation seen between the countries could be explained by the rest of the significant variables in the model. However, *Country* was significant (Table 4), which implies that neither temperature, nor old houses or the lack of designated tools etc. could explain all differences between countries. Some of the differences in prevalence levels between the countries may also be explained by the different sampling and testing schemes applied. Prevalence data from Denmark, Norway and the Netherlands were based on sock samples, whereas data from Spain, Poland, and the UK were based on cloacal swab samples. Sock samples are typically collected 7 to 10 days before slaughter while caecal samples are collected at the time of slaughter. Chowdhury et al. (2012) reported that the prevalence level decreased 9 percentage points from 23% for the years 2007–2009 to 14% for 2010. The differences between the sampling methods may be due to sock samples being less sensitive and/or to the extended time at risk before the cloacal swab samples are collected compared to the time at which the sock samples are collected. However, even when the differences in the sampling method are taken into account, there is still more variation between the countries than what could be explained by the variables in the model. The reason for this could be that some important factors which vary between the countries were not included in the study, or that the questions in the questionnaire may have been perceived in slightly different ways in the different countries, thereby introducing bias.

The interaction term *Temp∗EU* was significant. The reason for the differences in the effect of temperature between Denmark-Norway and the other countries could be that many other variables, known or unknown, have a relatively large impact on the *Campylobacter* prevalence for Spain, the Netherlands, Poland, and the UK and thereby weakens the relative effect of the temperature.

Thinning, where machines and staff enter the broiler house to catch part of the flock for slaughter, has been shown to be a risk factor (Berndtson et al., 1996; Hald et al., 2000) and it was also included as a variable (obtained from the questionnaire) in the present study. However, from several countries we only obtained *Campylobacter* status data collected before the first batch of broilers from a flock was slaughtered, and could therefore not analyze the effect of this practice.

4.3. Alternative model approaches

Other model approaches were tested to explore alternatives for categorizing the outcomes of some of the variables and including/excluding variables with a large number of missing values. Many of these alternative models found more or less the same significant variables as the main model. Two models, however, had slightly different results. One model did not include *Age of house∗Country* and *Age of house∗Tools* in the reduced model, but identified three other variables: *No. of houses∗Country, Water supply,* and *Rodent control frequency:* This alternative model was based on 3179 records, which were 807 records less than the main model. The variable *No. of houses* was modeled as a binary variable in the alternative model (categories: one broiler house versus two

and more broiler houses), rather than as a continuous variable (as in the main model). Having two or more houses increased the risk of introducing *Campylobacter* into a flock in Norway and Poland, whereas no difference was observed in Denmark, the Netherlands, and Spain. The UK had only farms with two or more broiler houses. For the variable *Rodent control frequency* the category with the least frequent rodent control (< 3 times a year) had a higher risk of introducing *Campylobacter* than those with more frequent rodent control (4–10 and 11–52 times a year). There were, however, not many observations with < 3 times a year. The *Water supply* showed an increased risk if farmers used surface water compared with private bore holes and municipal. Only Norway and Spain had a few farms (less than five out of 173 and 20 respectively) that used surface water and therefore the categories 'private bore holes' and 'surface water' were merged in the main model.

An alternative model was reached based on the reduced model described above. We continued the modelling process after having removed the *Rodent control frequency* since this variable had a large number of (systematic) missing values (20% of the data). The alternative reduced model was very similar to the main model except that *Rodent control frequency* was out and instead *Age of house*∗*Country* and *Age of house*∗*Tools* were in the model. This alternative reduced model was now based on 3975 records, only 11 records away from the main model.

Since Norway did not have prevalence data for the winter period, we examined the effect of not including Norway in the common EU model. This resulted in the same significant variables, only the *p*-values changed slightly. The number of data decreased from 3986 to 2355 data records. Hence, including Norway in the model did not bias the results even though the sampling period for Norway only covered the 'summer' period (from May to October).

4.4. Comparison with results from other studies

When comparing results from Denmark and Norway (Høg et al., 2016) with the results presented in this study, which includes data from more countries as well as climate data, we identified many of the same significant variables. The study of Danish and Norwegian data, found four more significant variables: *No. of houses*∗*Country, Stocking density*∗*Country, Boot dip,* and *Water supply.* These variables and their interaction terms with *Country* were, however, not significant in the main model in the present study. *Boot dip* is a disinfecting bath at the broiler house entrance, where staff can dip and disinfect boots prior to entering the house. In Denmark the risk of *Campylobacter* decreased on farms without boot dip, whereas in the Netherlands and Norway there was no effect. Spain, Poland, and the UK had only one type of response – all farms used boot dip. All together, these facts likely caused *Boot dip* to be insignificant in the present study. The variable *Water supply* was very close to being significant, with the highest risk estimate for farms using surface water or private bore holes. If the number of houses were broken down as the binary variable described earlier the variable *No. of house*∗*Country* was significant in one of the alternative models. As in the work of Høg et al. (2016), Norway had an increased risk for farms having two or more broiler houses whereas Denmark did not. In the present study, Poland had an increased risk with more houses, but this was not the case for the Netherlands, the UK, and Spain. Furthermore, the present study identified *Tools* and three interaction terms *Age of house*∗*Country, Age of house*∗*Anteroom+barrier,* and *Age of house*∗*Tools* to be significant risk factors.

A previous Danish study with data from 1999–2000 identified the risk factors: *Age of house, Rodent control, Age of chicken when introducing whole wheat, Age of chicken at slaughter, Storage of wheat, No. of chimneys, No. of broiler houses,* and *Density of cattle farms in the area* (Sommer et al., 2013). However, many of the variables in the two models are different, which make comparison of the studies difficult. Nevertheless, some of the variables were alike and were identified in both studies such as *Age of broiler house, Rodent control,* and *No. of houses.* Variables concerning the use of wheat in the feed, no. of chimneys in the broiler houses, and presence of cattle farms were not part of the present study and could therefore not be identified as risk factors. *No. of chimneys* were interpreted as being related to how open the house was to the environment allowing insects to enter the broiler house. Variables on boot dip, tools, and downtime were not part of the study of Sommer et al. (2013). Variables on drinkers and water supply were, however, included but found not to be significant in the study from 1999–2000. In both studies of Danish broiler farms (data from1999–2000 and 2010–2011) the following variables were found not to be significant: other animals on the farm, ventilation system, surrounding area, and number of persons with access to the broiler house.

Chowdhury et al. (2012) also analyzed Danish broiler farms from the period 2009–2010. In accordance with the Danish studies mentioned above they found that the age of the broiler house and the age of chicken at slaughter to be significant risk factors. However, in contrast to the other Danish studies Chowdhury et al. (2012) also found the number of persons with access to the broiler house to be significant similar to what Guerin et al. (2007a) found. The oldest of the Danish studies identified *No. of houses* to be significant which was not the case for the later Danish studies. The contamination between houses may have been reduced between the times of the two studies due to increased focus on the biosecurity. Since 2000 a control strategy for *Campylobacter* in broilers has been implemented on all farms through a quality assurance schemes (Rosenquist et al., 2009).

The risk factors identified in this cross country study resemble risk factors that have previously been published in single country studies. Despite different study designs, many studies have found risk factors associated with the level of biosecurity on the broiler farms and in the broiler houses. Hence, hygiene barriers, no. of houses, season/temperature at the time of rearing, disinfection of boot dips, no. of staff, and other animals on farm or on neighboring farms are all among risk factors that have been identified in other studies (Adkin et al., 2006; Agunos et al., 2014; Ellis-Iversen et al., 2009; Guerin et al., 2007a; Guerin et al., 2007b; Guerin et al., 2008; Hald et al., 2000; Hald et al., 2007; Hansson et al., 2010; Newell et al., 2011). The analyses and results of the many risk factors studies carried out for *Campylobacter* in broilers have illustrated the complexity of *Campylobacter* epidemiology and explain why there is no single simple method to prevent the broilers from becoming colonized with *Campylobacter.* Nonetheless, controlling *Campylobacter* in indoor conventional broilers is primarily a question of strict management practices with a high level of biosecurity and broiler houses that are closed off to the environment. However, as illustrated by the different studies, there are many factors involved in achieving a high level of biosecurity and some are more important than others (Adkin et al., 2006; Agunos et al., 2014; WHO, 2012).

Messages from the present work to future risk factor studies are: Improve the quality of the data, aim at having representative data with no missing values, use broiler house as the unit of the analysis rather than farm, and add optionally new explanatory variables and skip others. One way to facilitate this would be to carry out a thorough pilot questionnaire survey to test the responses to the drafted questions. Also, using electronic questionnaires that can be answered on-line, would greatly facilitate collecting data for such surveys.

4. Conclusion

In conclusion, we have shown that the involved six countries across Europe share the same risk factors for conventional, indoor broilers becoming colonized by *Campylobacter* despite differences in their broiler production and climates. Identified risk factors were broiler houses older than 15 years, absence of anterooms and barriers in each house, shared tools between houses, as well as a long downtime and drinker systems with water reservoirs such as bells or cups. All of the identified risk factors were somehow related to inadequate practice of biosecurity on the farms. Hence, maintenance of strict biosecurity in broiler houses will inevitable reduce the *Campylobacter* prevalence in broilers. However, the broiler flock prevalence was also influenced by temperature and by other, unknown country specific factors.

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