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ORIGINAL ARTICLE

Modelling-based identification of factors influencing campylobacters in chicken broiler houses and on carcasses sampled after processing and chilling

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Abstract

Aims: To identify production and processing practices that might reduce *Campylobacter* numbers contaminating chicken broiler carcasses.
Methods and Results: The numbers of campylobacters were determined on carcass neck skins after processing or in broiler house litter samples. Supplementary information that described farm layouts, farming conditions for individual flocks, the slaughterhouse layouts and operating conditions inside plants was collected, matched with each *Campylobacter* test result. Statistical models predicting the numbers of campylobacters on neck skins and in litter were constructed. Carcass microbial contamination was more strongly influenced by on-farm production practices compared with slaughterhouse activities. We observed correlations between the chilling, washing and defeathering stages of processing and the numbers of campylobacters on carcasses. There were factors on farm that also correlated with numbers of campylobacters in litter. These included bird gender, the exclusion of dogs from houses, beetle presence in the house litter and the materials used to construct the house frame.
Conclusions: Changes in farming practices have greater potential for reducing chicken carcass microbial contamination compared with processing interventions.
Significance and Impact of the Study: Routine commercial practices were identified that were correlated with lowered numbers of campylobacters. Consequently, these practices are likely to be both cost-effective and suitable for adoption into established farms and commercial processing.

Introduction

In 2008, the European Union (EU) undertook a survey of campylobacters in chicken broiler meat in 26 member states, Norway and Switzerland (Anonymous 2010). The United Kingdom was ranked tenth worst in terms of absolute *Campylobacter* prevalence (75% of samples tested positive); approximately 67% of the samples had >10 colony-forming units (CFU) campylobacters per g of meat. The findings of the EU survey agreed broadly with earlier surveillance by Adak *et al.* (2005) who also estimated that over 280 000 cases of campylobacteriosis and 191 deaths were caused annually in England and Wales as a consequence of *Campylobacter*-contaminated poultry meat (Adak *et al.* 2005).

In response to the EU survey findings, a working group with members drawn from the UK government, the British Poultry Council (a poultry processor trade association), and the British Retail Consortium (a trade association for larger retailers) was established. The

purpose of this Joint Working Group (JWG) was to reduce the numbers of campylobacters in British poultry meat and to undertake continuous monitoring of processed broiler samples postchill in slaughterhouses as a way of monitoring progress towards a *Campylobacter* reduction target. The UK target for reduction of *Campylobacter* was a reduction in the percentage of chickens that had the highest contamination (>1000 CFU per g), from the EU-measured baseline of 27% in 2008 to 10% by the end of 2015, with measurement undertaken after carcass chilling.

Of particular interest to the JWG was one of the recommendations of the EFSA Baseline *Campylobacter* in Broilers analyses report (Anonymous 2010). In brief, the EU-wide survey identified that some slaughterhouses were better at removing campylobacters from broiler carcasses compared with others. Consequently, it was recommended that 'further national studies to identify more closely, at batch- and slaughterhouse- level, the factors that put broiler batches and carcasses at risk of becoming respectively colonized or contaminated with *Campylobacter* in a country'. This report outlines the findings of work aimed at the identification of poultry risk factors for campylobacters in broiler meat in the United Kingdom.

The statistical approach chosen for risk factor identification was multilevel modelling. The methodology was chosen because it extends traditional statistical techniques to take appropriate account of population context. Batches of broilers held in sheds on farm have a hierarchical, or nested structure because consecutive batches of birds are raised in the same sheds on the same farms and are potentially exposed to similar or identical risk factors. These same birds are further exposed to similar or identical risk factors during processing in one of the several participating slaughterhouses. Much of the mathematics relating to multilevel statistical analyses was developed by social scientists (Nuttal *et al.* 1989). A common example in the literature is the statistical analyses of multiyear data describing consecutive years of students in classrooms within schools (Nuttal *et al.* 1989), which has a high degree of analogy with consecutive batches of birds in broiler houses on farms. In order to fully account for the fact that different batches of birds are not fully independent from flocks raised previously in the same farm environments and processed in a slaughterhouse environment, specific consideration of the data hierarchy is required (Nuttal *et al.* 1989). A multilevel modelling approach fulfils the required specific consideration because the analysis undertakes an evaluation of the hierarchical data structures simultaneously and makes redundant any uncertainties relating to the depth of analyses, which can plague single-level models such as multiple regressions (Rasbash *et al.* 2009).

Materials and methods

Neck skin test sample

Excision-based sampling of neck skins was undertaken on moving lines during normal commercial processing. Sample collection was immediately after the chilling phase of processing. Sample collection involved turning a sterile 304 mm × 177 mm stomacher bag (Seward, Thetford, UK) inside out over a gloved hand, selecting carcases with neck skins of an appropriate length and excising a 10-g sample using a pair of sterile scissors into the stomacher bag. Three combined neck skins were processed as a single sample. Typically, samples were collected once-to-twice per month from 19 processing lines situated in 16 different processing plants in England, Scotland, N. Ireland and Wales.

Microbiological testing to determine *Campylobacter* numbers on broiler neck skins

Nine volumes of maximum recovery diluent (MRD; Oxoid, Basingstoke, UK) were added to each sample before homogenization for 1 min using a stomacher (model number BA 6021; Seward). *Campylobacter* were enumerated using the ISO 10272-1:2006 standard method. All decimal dilutions were made using MRD and plating was onto modified charcoal cefoperazone desoxycholate agar (mCCDA; Oxoid). Incubation was under microaerobic conditions (CampyGen; Oxoid) at 41·5°C for 48 h. Confirmation of *Campylobacter* spp. was by microscopic examination of five colonies per plate to confirm corkscrew motility; an inability to show visible growth at 25°C after 72 h, positive testing for oxidase and the inability to ferment lactose and sucrose. Bacterial numbers on all decimally diluted plates were converted into CFU per g or CFU per ml as appropriate according to the criteria described by ISO 6887-1:1999.

Broiler house litter test sample

Broiler flocks are typically partly depopulated to create space in houses as the birds grow. Samples were collected from flocks not more than 12 h before birds were partly or fully depopulated from housing. Typically, each flock was sampled two or three times, although no further samples were collected once a flock became colonized. Detailed sample collection instructions have been previously published (Madden *et al.* 2014). In brief, the sample tested was collected by farm employees wearing polythene

covers (Bowden and Knights, Thetford, UK) over their boots to prevent their footwear from contaminating the sampling swabs. Sampling was using disposable Tyvek overshoes (Arco, Hull, UK) with samplers walking up and down the entire length of the house. The numbers of campylobacters in litter-derived samples were determined using quantitative polymerase chain reaction (qPCR).

DNA extraction

The sample collection overshoes were homogenized into 50 ml of MRD for 1 min using a stomacher (Seward 400). DNA in the MRD was purified according to the manufacturer's instructions using an automated QIAxtractor robot (Qiagen, Manchester, UK), DX reagents (Qiagen, 950107) and QIAxtractor DNA plasticware (Qiagen, 950037). Final elution was into 150 μl of buffer (10 mmol l^{-1} Tris-HCL (pH 8·5, 0·5 mmol l^{-1} EDTA)). Positive and negative extraction controls were included. A QIAgility robot (Qiagen) loaded the extracted samples into 96-well real-time PCR plates (Life Technologies, Waltham, MA).

Quantitative polymerase chain reaction

Real-time PCR was performed using the Mericon *Campylobacter* spp. detection kit (Qiagen). Each qPCR reaction was supplied from the manufacturer as a lyophilized mix of primers and labelled probe, which was reconstituted as a master mix. Five microlitres of the master mix was aliquoted into each test well of a 96-well plate using a QIAgility robot. qPCR plates were sealed with adhesive film (MicroAmp Optical Adhesive Film' Life Technologies) and briefly centrifuged (5000 ***g***, 30 s) before thermal cycling. Reactions were undertaken on an ABI7500 (Applied Biosystems, Warrington, UK) instrument running 7500 fast systems sequence detection software (ver. 1.4.0.27). The instrument cycling conditions were an initial heat to 95°C for 5 min to activate the HotStarTaq Plus DNA Polymerase. This was followed by 40 cycles of a three-step amplification cycling: denaturation, 15 s at 95°C; annealing, 23 s at 60°C, with data collection at 60°C; extension, 10 s at 72°C. Detection reporter excitation and emission channels for *Campylobacter* DNA were 495 and 520 nm respectively. The internal controls used excitation at 524 nm and detection at 557 nm. A calibration curve was constructed by plotting the detection cycle values of the *Campylobacter* DNA standards against known numbers of cells determined by traditional culture as described above. The range spanned six decimal dilutions of a *Campylobacter* type culture (1×10^7 to 1×10^2 CFU per ml). At least five separate amplifications were used for each point on the standard curve. The standard curve was used to convert the detection cycle to numbers of campylobacters.

Questionnaires matched to each test result

For each test result collected, food business operators were requested to provide a corresponding amount of supplementary information for use in the identification of risk factors for bird colonization by *Campylobacter*. The entire questionnaires are provided as Figs S1–S5. In brief, the additional information comprised as follows.

Farm infrastructure (Fig. S1); which asked questions relating to a farm on one occasion. The answers to these questions were unlikely to change (e.g. What was the farm postcode? What was the farm County Parish Holding number (or other UK government-issued unique farm identifier)?).

Flock Information (Fig. S2); which asked questions that were batch-specific and described the conditions during the farming of the birds (e.g. Were antibiotics given to the birds? How long was the shed empty before populating with the current batch of birds?). These questions were asked for each batch of birds processed that was tested, because the answers could differ between batches.

Plant infrastructure (Fig. S3); which asked questions as a way of describing the basic layout and operations of the plant. These questions were also asked only once. As for the farm infrastructure, the answers to most of these questions were unlikely to change (e.g. Was bird stunning by gas or electricity? How many plucker banks did the line have?).

Plant operations during the processing of individual slaughter batches (Fig. S4). These questions related to processing conditions in the plant at the time the tested batch of birds were slaughtered and processed (e.g. What was the scald tank temperature? How long were the birds held in lairage before shackling to the line?). These questions were also asked for each batch of birds processed, because the answers could differ for each batch.

Plant operations on sampling day (Fig. S5); which asked questions relating to processing conditions in the plant on the day of slaughter and was designed to minimize duplicate information where multiple batches of birds were sampled in a single processing day (e.g. How many days was it since the chiller was last cleaned?). These questions were asked once for each day that a batch of birds was sampled.

Development of the on-line data collection and reporting facilities

The questionnaires were converted into web forms and systems were established to allow processors to securely

log in to a Website located on a subdomain of the former FSA Website using the URL (universal resource locator) www.ukmeat.org. The recruitment process was that processors were informed about the study from a variety of sources such as the British Poultry Council, the Food Standards Agency, an industry-government steering committee called the JWG and routine visits to poultry slaughterhouses made by the researchers. Processor technical staff were given the option of using a web browser to visit the Website and enter data directly or return paper copies of the questionnaires. Those that agreed to participate were assigned a username and password that allowed them to supply some basic descriptions of their operations, including a valid email address and a mobile phone number. The provided email address was verified by sending an activation link for the logon details. Initially, the inclusion criteria were chiefly that the slaughterhouse and associated farms were willing to provide information describing their farms and flocks for *en masse* anonymized risk factor identification.

The technical details of the Website were that it was built using the Microsoft (MS; Redmond, WA) ASP.NET framework ver. 2.5 on a webserver running the MS Server 2008 operating system. Customized active webpages were coded using either the C#.NET or VB.NET programming languages, and the questionnaire–response data were collected and stored in an instance of the MS-SQL (structured query language) database program version 2008. Variable names corresponding to the supporting information questionnaires are provided as Tables S1–S5. All data saved and retrieved from the database were as parameterized, HTML-en/de-coded queries that prevented malicious script injection into the database and unintended manipulation of the page script. Attempts were made to make the site as easy to use as possible for processors (e.g. the number of response columns in the online questionnaire for the shed detail data were dynamically created in response to a previous question that asked 'How many sheds were there on the farm?').

Modelling to identify factors that predicted the numbers of campylobacters on broiler neck skins

The information in the relational database was required to be combined into a single flat-form spreadsheet as preparation for analyses. For each slaughter batch, the corresponding farm information was linked to it to form a new single data table. A number of slaughterhouse operators take birds from farms typically in a repeating cycle of between 45 and 55 days. Thus, there were instances where different batches of birds were taken from the same farms on different days. In these cases, a complete set of farm infrastructure data was included for every instance of birds. Farmed batches of birds were linked based on farm CPH number, the plant operations identifier (formerly known as the plant licence number) and the bird harvest date.

Information collected by the Website was held in the database tables as standard text. The first stage of modelling was to encode the text responses into numeric information (e.g. House construction frame wood = 1, House construction frame metal = 2). The two main types of variable were defined as ordered categorical or nominal. Ordered categorical information bore some relation to the encoded number. For example, the first clearance of birds from a house was encoded as '1', the second removal of birds as '2'. For nominal variables, there was no relationship between the value of the number and the information encoded. Continuous data such as bird age in days or house age in years was used without further conversion. Data were sorted in MS Excel (ver. 2010) by slaughterhouse identifier, then farm identifier and then shed identifier prior to import into the modelling software, as a formatting requirement of the software.

The software package MLwiN (Rasbash *et al.* 2009) was used to construct a hierarchical linear model to account properly for the correlation structure within the collected data. In the initial Poisson model, a two-level hierarchy was specified as the slaughterhouse and supply farm identifiers. In subsequent models, a broiler house identifier was included as a third level in a model that included subsequent batches of birds. For all of the models developed, the assumptions necessary for fitting models of this type (e.g. normally distributed residuals and homogeneity of their variance) were verified as satisfactory. The model was developed starting from a base model that included a constant and a categorical variable which specified an additive effect for each sample collected. The modelling process proceeded by alternately fitting predictor variables to a model that attempted to predict the log numbers of campylobacters and removing those that were not significant at $\alpha \leq 0{\cdot}05$, using a chi-squared test of the change in likelihood (Wald test). Variables that had been removed were then retested in later iterations as the model was developed until only statistically significant predictor variables remained.

Modelling to identify factors that predicted the numbers of *Campylobacters* in broiler house litter

A near-identical approach was taken to attempt to identify risk factors for flock colonization on farm. For the farm models, only the farm infrastructure and flock information questionnaires were used to attempt to predict the numbers of campylobacters in litter as described above.

Results

Data manipulations prior to analyses

Critical inspection of the raw data revealed there were issues that were required to be resolved prior to multivariate statistical analyses. The most common issue was an inadvertent duplication of data entry. Overall there were 23 instances of identical results being entered for the batch farming conditions ($n = 13$), individual slaughter batch questionnaires ($n = 8$) and processing conditions on the day of slaughter ($n = 2$). In some ($n = 5$) cases, there were minor differences in a small number of question responses in otherwise duplicated returns. The most-recently saved version was used in these instances. After parsing to remove duplicate data, the data set was imported into the MLwiN software for statistical analyses.

After the removal of data that did not have a *Campylobacter* count above the limit of detection of the test method (10 CFU per g), 964 rows of information remained. These data were used to construct a basic model in which only well-established, seasonal variation in colonization was included. The results of the initial model are shown as Fig. 1.

An important finding from the initial model was that it quantified the variance components described by the data after seasonal variation influencing the numbers of campylobacters on neck skins in processing plants had been removed. Around 23·7% (0·210/0·886) of the observed variation was from factors associated with the slaughterhouse, with the remaining variance (76·3%; 0·676/0·886) associated with on-farm agricultural factors. Thus, although it was reported previously that slaughterhouses differ in their ability to hygienically process colonized birds into carcasses (Anonymous, 2010b), what occurred during the agriculture of the birds on farm was more than three times as important in terms of the final carcass *Campylobacter* load.

The initial model (Fig. 1) was expanded by the addition and removal of factors in an attempt to account for the observed variation. A particular emphasis was placed on slaughterhouse factors because, for the initial data collections, there was a more comprehensive data set associated with birds during processing compared with rearing. The disparity in completed questionnaires was a consequence of information being more difficult to obtain from farms compared with process data that was readily-available to plant personnel. The final best-fit Poisson model is shown as Fig. 2. The processing stages that had a significant influence on *Campylobacter* numbers on neck skins included chilling, washing and the removal of feathers.

A Poisson model identifying risk factors for the colonization of broiler chickens on independent farms

Since the farm phase was identified by the initial model as important in contributing to the variation in counts, further analysis was carried out to identify on-farm risk factors. A significant difference between the initial postchill neck skin work and the farm-focussed work was that a litter-derived sample was used to assess the bird status and degree of any colonization. Two different categories of farm were identified and investigated independently for risk factors specific to each type. These were firstly, smaller, independent farms supplying lower throughput integrated or independent processors. Typical processor line speeds were 4000–5000 birds per hour. Secondly, integrated farms owned by high-throughput (10 000–12 000 birds per h) processing plants, were also examined.

The small farm model calculated the terms exerting significant influence on the $\log_{10}$ *Campylobacter* count

$$\text{LogCount}_{ij} \sim N(XB, \Omega)$$

$$\text{LogCount}_{ij} = \beta_{0ij}\,\text{Const} + -0{\cdot}183073(0{\cdot}047013)\text{Sin}_{ij} + 0{\cdot}093399(0{\cdot}044073)\text{Cos}_{ij} + -0{\cdot}000329(0{\cdot}000168)\text{TofYear\^{}1}_{ij}$$

$$\beta_{0ij} = 3{\cdot}104814(0{\cdot}161227) + u_{0j} + e_{0ij}$$

$$[u_{0j}] \sim N(0, \Omega_u) : \Omega_u = [0{\cdot}209683(0{\cdot}079081)]$$

$$[e_{0ij}] \sim N(0, \Omega_e) : \Omega_e = [0{\cdot}676261(0{\cdot}031085)]$$

*–2*loglikelihood(IGLS Deviance)* = 2404·186035(964 of 1077 cases in use)

Figure 1 The results of an initial run of a Poisson multilevel model prior to the addition of any potential risk factors to explain variance. Suffixes *i*, and *j* refer to farms and slaughterhouses respectively. The model simply calculated the seasonal change with time of year (expressed as sin and cos transformation of sequential day number mapped to a 360° scale, together with a linear effect (decrease) from the start to the end of the year), and their significant influence on the $\log_{10}$ *Campylobacter* count on carcass neck skins. β_0 is the constant in the equation.

and is shown in Fig. 3. The constant in the equation is tied to the first category of each categorical variable and to a bird age of zero. Thus, for example, to calculate the predicted (mean) count for a flock of age 42 days, of bird gender category 3 (mixed gender birds), in a house with house construction category 2 (metal framework), but otherwise within the first group of the remaining categorical variables, the equation shown as Fig. 4 would be used.

Overall, the analysis in Fig. 3 showed that between an age of 26–50 days, for every 1-day increase in the age of a flock there was a mean increase in $\log_{10}$ *Campylobacter* litter counts of 0·331 CFU per g ($P < 0·001$). There was also an overall protective effect for some bird genders. Houses containing exclusively female birds had a geometric mean that was 1·107 $\log_{10}$ CFU per g lower compared with sheds containing male gender birds ($P < 0·001$). Mixed gender sheds also tended to have significantly lower counts of 0·785 $\log_{10}$ CFU per g compared with sheds containing only male birds ($P = 0·020$). There was also an overall effect of the type of house construction on campylobacter numbers with those broiler houses constructed from metal frames having 0·462 $\log_{10}$ counts greater than those with wooden frames ($P < 0·001$). If prebiotics were fed to birds, the mean $\log_{10}$ count was increased by 1·400 ($P < 0·001$).

Farm category (Table S6) also exerted an influence on $\log_{10}$ *Campylobacter* numbers. More specifically,

$$\text{LogCount}_{ij} \sim N(XB, \Omega)$$

$$\begin{aligned}\text{LogCount}_{ij} = {} & \beta_{0ij}\,\text{Const} + 0·3954526(0·2794164)\text{IOCorrectiveActionsCompletionTime}_{ij} \\ & + 0·2345036(0·1140700)\text{PostChillCTMZ}_{ij} \\ & + 0·1334945(0·0437489)\text{PluckEffectivenessCriteriaMet}_{ij} \\ & + -0·2308706(0·1030306)\text{ChillerCleanFreqMet}_{ij} + -0·1890836(0·0472284)\text{Sin}_{ij} \\ & + 0·1163740(0·0443917)\text{Cos}_{ij} + -0.0003337(0·0001676)\text{TofYear}_{ij}\end{aligned}$$

$$\beta_{0ij} = 2·9792702(0·2853364) + u_{0j} + e_{0ij}$$

$$[u_{0j}] \sim N(0, \Omega_u) : \Omega_u = [0·1572533(0·0607311)]$$

$$[e_{0ij}] \sim N(0, \Omega_e) : \Omega_e = [0·6634510(0·0306290)]$$

*–2*loglikelihood(IGLS Deviance)* = 2361·8461608(956 of 1077 cases in use)

Figure 2 A multilevel Poisson model describing the sources of variance influencing carcass neck skin *Campylobacter* load in UK slaughterhouses. The standard errors of the estimates are shown within the brackets after the parameter estimates. The statistical significance of the individual terms can be calculated by dividing the parameter estimate by its standard error and referring the result to a normal distribution. Suffixes *i* and *j* refer to farms and slaughterhouses respectively. The temporal components shown in the model in Fig. 1 have been retained. The constant (β_0) in the equation is tied to the first category of each of the categorical variables. Definitions for variable names are provided in Tables S1–S5.

$$\text{LogLoading}_{ijk} \sim N(XB, \Omega)$$

$$\begin{aligned}\text{LogLoading}_{ijk} = {} & \beta_{0ijk}\text{Constant} + 0·331(0·013)\text{BirdAge}_{ijk} + -1·107(0·277)\text{BirdGender\_2}_{ijk} \\ & + -0·785(0·339)\text{BirdGender\_3}_{ijk} + 1·400(0·360)\text{chkPrebiotic\_1}_{ijk} \\ & + -1·091(0·422)\text{NewFmCat\_2}_{k} + -0·477(0·986)\text{NewFmCat\_5}_{k} \\ & + 2·459(0·448)\text{NewFmCat\_6}_{k} + -0·263(1·036)\text{NewFmCat\_7}_{k} \\ & + 0·807(1·646)\text{NewFmCat\_10}_{k} + -2·249(1·519)\text{NewFmCat\_11}_{k} \\ & + 1·789(0·962)\text{NewFmCat\_13}_{k} + -1·237(0·433)\text{NewFmCat\_14}_{k} \\ & + 0·462(0·175)\text{HouseConstruction\_2}_{jk} + 1·438(0·843)\text{HouseConstruction\_3}_{jk}\end{aligned}$$

$$\beta_{0ijk} = -8·561(0·521) + v_{0k} + u_{0jk} + e_{0ijk}$$

*–2*loglikelihood(IGLS Deviance)* = 7616·578(1780 of 1780 cases in use)

Figure 3 The final model for independent farms produced from the MLwiN multilevel analysis. The standard errors are shown within the brackets after the parameter estimates. The statistical significance of the individual terms can be calculated by dividing the parameter estimate by its standard error and referring the result to a normal distribution. Suffixes *i*, *j* and *k* refer to batch, broiler house and farm respectively. Farm categories (NewFmCat_x) are listed in Table S6. Definitions for variable names are provided in Tables S1–S5. The constant (β_0) in the equation is tied to the first category of each categorical variable and to a bird age of zero.

compared with independent farms supplying independent processors, independent farms supplying integrated processors had lower counts in their litter by around 1·091 $\log_{10}$ CFU per g ($P = 0{\cdot}001$). Furthermore, there were two categories of farms supplying two different independent processors that had counts which were 2·459 $\log_{10}$ CFU per g lower ($P = 0{\cdot}001$) and 1·237 $\log_{10}$ CFU per g lower ($P < 0{\cdot}001$) than a general group of independent farms supplying independent processors. The remaining categories of farm were not significantly different from category 1 (independent farms supplying independent processors), although an elevated $\log_{10}$ count for the category 13 farms (a mix of farms supplying a single specific independent processor) that was 1·789 higher and only just failed to reach statistical significance ($P = 0{\cdot}062$).

A Poisson model to determine risk factors for bird colonization on integrated farms

For the integrated farm model, litter testing and supplementary data collections were undertaken from at least three different batches of birds from sheds on 50 different farms. There were 876 sets of supplementary information collected and the participating farms sent birds to eight processing lines in seven different slaughterhouses. The integrated farm model had two levels of hierarchy at the farm and house levels. The farm level explained 32·9% of the observed variation with the remaining 67·1% variance associated with individual broiler houses/flocks. The final model is shown as Fig. 5.

$$\text{Log}_{10}\,\text{count} = -8{\cdot}561 + (0{\cdot}331 \times 40\ (\text{days})) - 0{\cdot}785 + 0{\cdot}462$$

Figure 4 An example of the use of the equation shown in Fig. 3. The calculation shows the predicted (mean) $\log_{10}$ *Campylobacter* count for a flock of age 40 days for mixed gender birds (BirdGender_3; 0·785), in a metal framed broiler house (HouseConstruction_2; 0·462).

Discussion

The processing model (Fig. 2) revealed a number of variables that had significant association with the numbers of campylobacters contaminating postchill neck skins. It had been previously reported that effective cold water washing caused typical reductions in *Campylobacter* numbers from 2·58 log CFU per ml chicken carcass rinse to 1·15 log CFU per ml (Berrang and Bailey 2009). Consequently, it was no surprise that early iterations of the model highlighted the importance of rapid repair of the inside outside (IO) washer were it to fail or operate inefficiently during processing. However, in the final version of the model, the time taken to implement corrective actions to ineffective IO washing fell just outside the threshold for significant correlation. However, there were other factors that collectively remained significant. These included the postchill carcass temperature ($P = 0{\cdot}032$); with lower temperatures, which was associated with fewer campylobacters. It is also established that freezing chickens (Haughton *et al.* 2012) and chicken meat (Harrison *et al.*

$$\begin{aligned}
&\text{Log10Campy}_{ij} \sim \text{N}(XB, \Omega)\\
&\text{Log10Campy}_{ij} = \beta_{0ij}\,\text{Constant} + 0{\cdot}087(0{\cdot}015)\text{BirdAge}_{ij} + -0{\cdot}826(0{\cdot}303)\text{No}_{ij}\\
&\qquad + -0{\cdot}761(0{\cdot}582)\text{Yes}_{ij} + -0{\cdot}808(0{\cdot}190)\text{BeetlePresence}_{ij}\\
&\qquad + 0{\cdot}032(0{\cdot}008)\text{HouseAge}_{ij} + -0{\cdot}510(0{\cdot}163)\text{BootsDipped}_{ij}\\
&\qquad + 0{\cdot}319(0{\cdot}082)\text{SampleType}_{ij}\\
&\beta_{0ij} = 0{\cdot}314(0{\cdot}673) + u_{0j} + e_{0ij}\\
&[u_{0j}] \sim \text{N}(0, \Omega_u) : \Omega_u = [1{\cdot}879(0{\cdot}450)]\\
&[e_{0ij}] \sim \text{N}(0, \Omega_e) : \Omega_e = [3{\cdot}818(0{\cdot}188)]
\end{aligned}$$

Figure 5 The final model produced for integrated farms by the MLwiN multilevel analysis. The standard errors are shown within the brackets after the parameter estimates. The statistical significance of the individual terms can be calculated by dividing the parameter estimate by its standard error and referring the result to a normal distribution. Suffixes *i* and *j* refer to broiler house and farm. The constant (β_0) in the equation is tied to the first category of each categorical variable and to a bird age of zero. The model describes how the significant variables (Tables S1–S5) exert influence on the $\log_{10}$ *Campylobacter* count, for example overall, the analysis in Fig. 5 showed that between the ages of 26–50 days, for every 1-day increase in the age of a flock there was a mean increase in $\log_{10}$ *Campylobacter* litter counts of 0·087 CFU per g litter ($P < 0{\cdot}001$).

2013) can lower campylobacter numbers. In some UK plants chilling that is effective enough to turn neck skins hard with visible surface ice formation occurs, especially when processing larger birds, which may necessitate a slower line speed and consequent increased chilling time. We consider it likely the exceptionally low temperatures achieved on these carcasses could cause freeze-mediated stress and cellular death. The likely mechanism operating for the rates of cooling found in commercial carcass chillers, is that freezing commenced in small isolated volumes of water (Archer 2004). The freezing of these small volumes caused dissolved solutes to be displaced into the surrounding unfrozen liquid, thereby increasing the osmotic potential of that fluid (Dumont *et al.* 2004). Osmosis, driven by hypertonic extracellular fluids would remove water from the cytoplasm of the campylobacters (Dumont *et al.* 2004). Ice crystals, formed from the water remaining inside the cytoplasm, are the primary method of cellular damage during freezing (Toner *et al.* 1990). However, when cooling rates of liquids are low (a few degrees per minute), it is possible that all of the intracellular water can be removed from the cell before ice crystals form (Dumont *et al.* 2004). In addition, there is evidence that superoxide radicals form during freezing, which are similarly concentrated in unfrozen extracellular liquids and consequently contribute towards the death of campylobacters (Stead and Park 2000).

Most poultry processing plants set themselves performance criteria for key processing stages that are aimed at maintaining effective processing. One common performance target is the effectiveness of plucking (defeathering). Plucking equipment is adjusted to take account of different bird breeds (shapes) and sizes. Effectiveness is assessed by the presence of faecal leakage after plucking and the numbers and degrees of carcass damage. Meeting the plucking effectiveness target was identified by the model as associated with reduced *Campylobacter* load on carcasses ($P = 0{\cdot}0082$). The finding is not surprising because the defeathering of chicken broiler carcasses has been identified as a high risk area for cross-contamination between birds and flocks (Allen *et al.* 2003a,b, 2008). Furthermore, *Campylobacter* contamination of carcasses after plucking is highest compared with all of the other processing stages (Aburuwaida *et al.* 1994; Hinton *et al.* 2004). Takahashi *et al.* (2006) further investigated the mechanisms of how the contamination of carcasses was increased using basic genetic fingerprinting of the campylobacters as an indication of source. Existing populations of campylobacters around the pluckers were shown to become more diverse as birds from different farms were processed. Furthermore, the new strain types observed were isolated more frequently after defeathering as compared to other processing steps. Takahashi *et al.* (2006) concluded that different strains of campylobacters were spread between carcasses sourced from different farms as a consequence of the plucking stage. Consequently, it seems reasonable to assume the model has identified that properly adjusted plucking equipment might minimize cross-contamination of campylobacters between different flocks (Takahashi *et al.* 2006).

The model also identified the chiller cleaning frequency being met as potentially important ($P = 0{\cdot}018$). Chiller cleaning is notoriously difficult to accomplish in poultry processing plants because the chillers are seldom empty and processing typically occurs 18–20 h per day. Air chilling is almost-exclusively used in broiler processing in the United Kingdom and there are reports that chilling can reduce the numbers of campylobacters measured from carcasses. Reductions in the numbers of campylobacters was observed in 6 of 10 batches of birds examined just before and just after chilling (Allen *et al.* 2008). However, although the observed reductions were significant ($P < 0{\cdot}001$), they were quite small and in only three of the positive batches was the reduction greater than one log. The authors remarked that the effect of chilling on carcass contamination was highly variable between different batches and also between different plants. Cross-contamination of campylobacters between carcasses in the chiller was suspected to be one of the reasons for the variable results, although the mechanism of spread was not pursued. Allen *et al.* (2008) also showed that chilling with added water sprays, which is commonly observed in British plants, can increase bacterial counts from the cavity of the carcass, especially for *Pseudomonas* spp. The use of water to aid chilling meant that some parts of the carcass were likely to retain enough moisture during storage to allow for survival of *Campylobacter* and also to withstand the drying process that occurs at the same time as chilling. However, although the model has identified higher chiller cleaning frequencies as correlated with lower numbers of campylobacters, there is currently little in the literature to fully explain any of the mechanisms operating.

There are a large number of reports on the seasonality of *Campylobacter* colonization of birds and also human illness (Chowdhury *et al.* 2013; Friedrich *et al.* 2016; Yun *et al.* 2016). Therefore, it was not surprising that the initial model (Fig. 2) included a highly significant sinusoidal pattern of change associated with season (the Sin and Cos terms in the model). The final information noted by the process model was that there was a significant linear trend of reducing the numbers of campylobacters across the entire study ($P = 0{\cdot}045$). The observed reduction was 0·0033 log CFU per g of neck skin per day.

The small farm model (Fig. 3) identified a significant association with campylobacters in house litter and some

bird gender categories. From a practical viewpoint on farm, both male and females are placed into houses on the same day and will generally be sourced from the same breeding stock (i.e. farmers tend to get males and females from the same parent flock). In the United Kingdom, around 50% of the day old chicks are delivered to independent farms with males and females mixed together.

Whether the gender and age predictors were correlated was investigated and it was determined that there was no relationship. Furthermore, there was no significant gender and age interaction influence on the change in *Campylobacter* numbers between the different gender categories. It is common in the United Kingdom for the lighter female birds to be cleared from houses first, with the males allowed to grow on to a greater weight. The underlying reason for that practice is because cocks have the capacity to grow to a heavier weight and also because cocks achieve a better feed conversion ratio (an index of how effectively the energy content of feed is converted to bird muscle) compared with pullets (young female chickens) (Howlider and Rose 1992; Abdullah *et al.* 2010). In the current statistical model, it was determined that although females were cleared in preference to males for roughly half of the time, there were also some processors that would harvest males first if they reached a set target weight before the females, thereby potentially masking any effect for age by gender.

For each day a bird was farmed there was a mean increase in $\log_{10}$ *Campylobacter* numbers of 0·331 CFU per g litter and this finding was further investigated. In the United Kingdom, independent processors will undertake multiple depopulations before finally emptying a shed. In particular, independent farms with very large sheds, such as those containing more than 50 000 birds; might partially depopulate the sheds as many as six to eight times before final clearance. The employees undertaking catching are a risk factor for *Campylobacter* colonization by birds in a house (Allen *et al.* 2008; Hue *et al.* 2010). Thus, if catching occurs in large sheds many times before some birds are caught, then there is an increasing likelihood the remaining mostly male birds will become colonized with *Campylobacter*. Extended, multiple depopulations are a consequence of independent processors with a tendency to favour heavier birds because a higher percentage of carcasses tend to be boned out for breast fillets rather than sold whole. Typical independent bird target weights are between 1·35 kg live weight and 3·5 kg live weight. Limiting the number of clearances on independent farms is confounded by the relatively low processing capacity of the abattoirs they supply, compared with integrated slaughterhouses.

The material used to construct houses was also significantly associated with the numbers of campylobacters in litter. We hypothesize that steel frames are generally stronger than the equivalent timber ones. Consequently, steel frames can be used to construct larger sheds than timber-framed ones. Larger sheds can hold a larger number of birds, and so the protective effect of wood framing may simply be a proxy for the number of birds placed and the number of depopulations, stress events and exposure to catchers required to clear the shed. We also noted that in contrast to metal, unpainted and pressure stained wood is porous. Thus, it might generally be expected that an exposed porous wooden surface would provide a niche for campylobacters. An alternative possible explanation for wood being beneficial is that there are natural antimicrobial resins in wood, and pressure-impregnated preservatives have at least the potential to be antimicrobial (Willfor *et al.* 2004). The species of wood used and its structure in terms of knots, influence the distribution and concentration of antimicrobials contained with the structure (Willfor *et al.* 2004).

The use of prebiotics and their correlation with an increased numbers of campylobacters in litter is perhaps something that requires further investigation. Generally, prebiotics influence gut microbiota either by providing a nutrient preferentially to a specific group of bacteria or specifically inhibit the growth of some bacterial groups. However, prebiotics can also influence immunological targets and adhesion to gut columnar epithelia and thereby indirectly promoting or inhibiting bacterial populations (Pourabedin and Zhao 2015). Our investigations into the nature of the prebiotic usage correlation were limited for this study. Although a number of farmers responded that prebiotics had been used, there were too few specific products listed for robust analyses.

The integrated farm model identified bird age as a potential risk factor (Fig. 5). It has been reported previously that bird age was a significant predictor of bird colonization status. The review by Sahin *et al.* (2015) reported that there are at least six previous reports of correlations between bird age and *Campylobacter* colonization. A number of explanations have been proposed including the breaking of shed biosecurity by the chicken catchers, seasonal and climate changes, the use of house ventilation fans during summer, the number of broiler houses on farm and local fly populations for sheds that lacked fly screens (Sahin *et al.* 2015). Allen *et al.* (2008) reported that campylobacters were isolated from chicken catchers, their clothing, vehicles and equipment immediately after arrival on farms. The isolations were from different sets of catchers working for a variety of UK processors in the mid-2000s, which was compelling evidence that in the United Kingdom, historically at least; the breaking of biosecurity was credibly implicated with *Campylobacter* colonization and bird age.

Also related to biosecurity breaches, it was apparent from the questionnaire responses that on biosecure farms, some dogs were allowed entry into bird housing. Perhaps not surprisingly, farmers that excluded their dogs from the houses had significantly decreased numbers of campylobacters in the litter ($P < 0{\cdot}05$). In the final iteration of the integrated model, litter wetness was also initially significant. However, inclusion of 'dogs in sheds' into the model provided a better explanation of the observed variation and caused the degree of litter wetness to fall below the level of statistical significance ($P = 0{\cdot}06$). More generally, the physical presence of a dog on farm did not quite reach significance as a risk factor ($P = 0{\cdot}057$). The importance of dogs on bird colonization status has previously been identified as important in Dutch broiler farms. Bouwknegt *et al.* (2004) report an increased *Campylobacter* prevalence (i.e. the percentage of farms testing positive for campylobacters) for farms where dogs lived (Bouwknegt *et al.* 2004). Ellis-Iversen *et al.* (2012) more generally reported that animals such as cattle, dogs, wildlife and rodents were significantly associated with positive flocks. Other studies have reported that farms with nonpoultry livestock in close proximity (<2 km, Jonsson *et al.* 2012) and the density of nonpoultry farming operations near to broiler farms (Sommer *et al.* 2013) are risk factors. Although there are reports of other livestock increasing broiler colonization prevalence in the literature, none of the other animals (cat, cattle, turkey, sheep, pig, horse, llama) assessed by this study had a significant impact on the numbers of campylobacters in the litter. However, it was quite rare for llamas, turkey, sheep and horses to be found on broiler farms.

As might be expected for a basic biosecurity practice, increasing the frequency of dipping boots in disinfectant was significantly correlated with lowered numbers of campylobacters in house litter (Fig. 5). Campylobacters can be ubiquitous environmental inhabitants and in general, bacteria, including campylobacters, persist in niches where there is moisture (Pitkanen 2013). Ellis-Iverson *et al.* (2012) report that campylobacters are routinely isolated from standing water (e.g. puddles) on broiler farms, whereas the dust extracted from mechanically ventilated broiler houses also routinely contain campylobacters (Chinivasagam *et al.* 2009). Our finding is contrary to the observations of Hog *et al.* (2016) who report the presence of boot dips on Danish farms is a risk factor for flock colonization. However, Hog *et al.* (2016), when discussing their unexpected result, acknowledge previous studies of the type undertaken by Gibbens *et al.* (2001), who report that the frequency of replenishment of disinfectant in a boot dip should be at least twice weekly, otherwise there is no protective effect (Gibbens *et al.* 2001). Other studies have concluded that insufficient active disinfectant within a boot dip can act as a reservoir for campylobacters (McDowell *et al.* 2008).

Although increasing litter moisture content was a significant variable in the model until 'dogs in sheds' were included, the presence of darkling beetles (*Alphitobius diaperinus*; also commonly referred to as the lesser mealworm) in the litter was also significantly correlated with elevated *Campylobacter* numbers ($P < 0{\cdot}01$). A high degree of litter wetness has been reported as a risk factor for the colonization of broiler houses by darkling beetles (Japp *et al.* 2010). Thus, although litter wetness itself was excluded from the model in favour of dog access to sheds, indicators of high moisture content remained significant. Information in the literature is quite sparse on the role of beetles in spreading campylobacters, thus the finding is quite novel. One study artificially contaminated darkling beetles before feeding them to broilers (Hazeleger *et al.* 2008). The authors reported that campylobacters could be isolated from those birds that had consumed the artificially contaminated insects. However, artificially contaminated campylobacters did not persist for extended periods on the beetles and could not be isolated 1 week after inoculation (Hazeleger *et al.* 2008). The Hazeleger study also collected a limited number of naturally contaminated insects from a commercial broiler farm and showed that when birds consumed these insects they also became colonized, albeit at low levels. A general conclusion of the work was that *Campylobacter* can be transmitted via beetles and their larvae, and the authors emphasized it was a likely scenario that such transfers applied to successive rearing cycles.

Sample type was also identified as having an association with *Campylobacter* levels. Sample type is probably a proxy for bird age because the allowed responses of first clearance, second or later clearance and final clearance tend to occur at specific bird ages. The model reported that there was increased flock colonization with each subsequent clearance. A number of authors have suggested the basis for the increased risk is the breaking of biosecurity (Alali and Hofacre 2016; Battersby *et al.* 2016; Hog *et al.* 2016; Meunier *et al.* 2016). Allen *et al.* (2008) report *Campylobacter* isolations from staff that harvest chickens, their boot grips, clothing and equipment, the insides of the vehicles they travel in as well as the bird transport crates, modules and fork-lifting machinery.

The final association identified by the integrated model was house age, with older houses being more likely to contain colonized birds. Recently, Hog *et al.* (2016) identified the same risk factor, although as a binomial variable, comparing houses older and newer than 5 years. Hog *et al.* (2016) briefly discuss that the basis of their finding could be that older houses are more likely to be in poorer repair and thus have a lower standard of biosecurity. Although this seems plausible, our farm-based

observations are that older houses can have a less hygienic fundamental design and can be difficult to effectively clean and sanitize. For example, newer broiler houses with a modern design lack the horizontal roof spars common in older houses. The upper surface of the roof supports required a ladder to be cleaned and many older houses have a layer of dust on these supports that can be several years old. There are broiler houses still in operation in the United Kingdom that predate the identification of *Campylobacter* as a foodborne human pathogen. We consider it likely that improved hygienic design and ease of cleaning is at least partly responsible for any potential protective effect of modern broiler houses.

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Conflict of Interest

Gary Ford declares employment by the National Farmers' Union, an organization that promotes UK agriculture. The other authors declare no conflicts of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 The questions asked to farmers that were used to create a description of the farm layout, number of broiler sheds and other aspects of the farm infrastructure that would change infrequently.

Figure S2 The questions asked to farmers regarding farming conditions for each individual batch of farmed birds.

Figure S3 The slaughterhouse infrastructure questionnaire used to create a description of the infrastructure of each plant and the equipment used to process birds.

Figure S4 The questionnaire used to gather information on the plant environmental conditions during the processing of an individual slaughter batch of birds.

Figure S5 The plant environment conditions questionnaire used to create a description of conditions during a processing day.

Table S1 Column names and variable types for the farm infrastructure data.

Table S2 Column names and variable types for each farmed batch of birds' information.

Table S3 Column names and variable types for processing plant infrastructure.

Table S4 Column names and variable types for individual slaughter batch responses at the time of processing.

Table S5 Column names and variable types for individual slaughter batch responses on the day of processing.

Table S6 Farm categories used for the smaller independent farms supplying lower throughput processors.