Food Standards Agency
food.gov.uk

Project title: Monitoring of campylobacters in UK poultry slaughter batches and carcasses and the collection of information from primary production and processing for risk factor elucidation.

Project reference: FS241051A (M01056)

Final report date: November 2016

Report authors: M. L. Hutchison[*1,2], M. A. Tchórzewska[2], D. Harrison[2], J. E. L. Corry[2], M. J. Taylor[3], R. H. Madden[3], V. M. Allen[2] and T. G. Knowles[2]

Author affiliations: [1]Hutchison Scientific Ltd, Union Street, Cheddar, Somerset. BS27 3NA, UK.

[2]School of Veterinary Science, University of Bristol, Langford, BS40 5DU, UK.

[3]Agri-Food Biosciences Institute, Newforge Lane, Belfast, BT9 5PX, N. Ireland.

Corresponding author: Mike Hutchison: Phone: +44 1934 741115; E-mail: mh@hutchisonscientific.com

© Crown Copyright 2016

This report has been produced by Hutchison Scientific Ltd under a contract placed by the Food Standards Agency (the Agency). The views expressed herein are not necessarily those of the Agency. Hutchison Scientific Ltd warrants that all reasonable skill and care has been used in preparing this report. Notwithstanding this warranty, Hutchison Scientific Ltd shall not be under any liability for loss of profit, business, revenues or any special indirect or consequential damage of any nature whatsoever or loss of anticipated saving or for any increased costs sustained by the client or his or her servants or agents arising in any way whether directly or indirectly because of reliance on this report or of any error or defect in this report.

EXECUTIVE SUMMARY

In 2008, the European Union (EU) undertook a survey of campylobacters in chicken broiler meat in 26 member-states, Norway and Switzerland (Anonymous, 2011). The United Kingdom was ranked tenth worst in terms of absolute *Campylobacter* prevalence with 75% of samples testing positive. Poultry meat is a significant source of campylobacteriosis in humans and responsible for the majority of the 280,000 estimated cases of foodborne disease in the UK caused by the bacterium. The response of a joint industry and government working (JWG) group established to combat *Campylobacter* colonisation of chicken broilers was to commence a programme of research and intervention aimed at reducing colonisation of poultry with *Campylobacter*. In addition, a target for reduced numbers of colonised birds and lower numbers of campylobacters on carcasses was agreed.

This study was concerned with the identification of risk factors that influenced the numbers of *Campylobacter* on broiler chicken carcasses post-chill. There were four main strands to the study. The first was concerned with the type of broiler carcass sample that was tested for campylobacters. The previous surveillance organised by the EU used a combination of neck skin and neck extension (i.e. breast) skin to assess the degree of carcass contamination. However, in the UK, there was a strongly-expressed, industry preference that the sample used for statutory *Salmonella* testing – three pooled neck skins, be used. The basis of the industry preference was that the statutory sample had to be taken anyway and a portion of the same homogenised sample could be cost-effectively removed by the laboratory for *Campylobacter* testing. As part of this study, the implications of changing the sample type from neck and neck extension to three pooled neck skins was investigated. Neck skin was found to be more heavily contaminated with campylobacters than breast skin. The JWG-agreed reduction target involved reducing the percentage of heavily contaminated samples (>1000 cfu/g) from 27% to 10% by the end 2015. The reduction was set against what was considered possible from the results of the original EU baseline survey in the UK. A conclusion of this work was that a change of test sample to pooled neck skin would make it more difficult to achieve the JWG-agreed reduction.

A second purpose of this study was to undertake testing under commercial processing conditions and otherwise accumulate laboratory test results derived from neck skins from post-chill broiler carcasses. Most of

the tests were paid for by the UK poultry processing industry. Since the results were generated by more than twenty testing laboratories, there was a requirement to determine if the results were equivalent between labs. To assess the abilities of the labs, a proficiency testing (PT) scheme for counting numbers of campylobacters was established. Acceptable PT performance was required before test results could be accepted for use in the project. One use for the collected test results was as a performance indicator of progress towards the JWG target.

The third strand of the study was to collect supplementary information matched with the *Campylobacter* test results. The supplementary information described the physical layout of farms, the farming conditions for individual crops of birds, the physical layout of processing plants and the operating conditions inside those plants as birds were processed. The general strategy was to use the supplementary information to construct a statistical model that could predict the numbers of campylobacters on post-chill broiler neck skins. A model was constructed to predict *Campylobacter* numbers on post chill neck skins using data collected between November 2011 and March 2014. The model that revealed there were around nine units of variation in an initial dataset. The source of 2/9 of this variation was caused by environmental conditions in the plant at the time of processing. The remaining 7/9 of the observed variation was caused by on-farm factors. Thus, an important finding from this project was that on-farm factors were three and a half times more important than processing plant factors in influencing numbers of campylobacters on post-chill broiler neck skins. The factors influencing bird colonisation on farms were therefore further investigated using numbers of campylobacters in broiler house litter as model targets for prediction as the final work thread of the project.

Two farm models were constructed using litter test results and supplementary information collected between October 2014 and September 2015. Independent farms supplying low-throughput slaughterhouses and integrated farms owned and operated by high throughput processors were investigated separately. For independent farms, bird gender, age at slaughter, shed size and construction material were significant predictors of numbers of *Campylobacter* in litter. Feed-withdrawal time was initially also statistically significant, with longer times increasing the numbers of campylobacters. However, this effect was masked in later model iterations; possibly because samples representing higher risk, longer feed-withdrawal times were under-represented as a coincidental consequence of the types of farms that participated in the study. The

processing line speed in a typical low-throughput slaughterhouse is 5000-6000 birds per hour; however, in a larger-throughput plant, the line speed can be twice as fast. In addition, larger throughput plants can run for up to 20 hours per day. Investigations revealed that independent farms partly depopulate birds from houses up to eight times before the shed is completely cleared, largely because of the limited capacity of the smaller processing plants. In addition, on independent farms, numbers of campylobacters in litter from larger sheds constructed of steel tended to be higher than in smaller sheds made of wood, and this seemed to be related to the higher numbers of birds in the larger sheds. The higher numbers of birds in larger sheds required even more depopulations. The multiple breaking of shed biosecurity during depopulation was a likely explanation for the increased risk of high numbers of campylobacters in litter from older birds and in sheds constructed of steel.

For integrated farms supplying high-throughput slaughterhouses, the factors which increased numbers of campylobacters in litter were identified to be higher bird age, the exclusion of dogs from broiler houses, the presence of darkling beetles (which are an indirect indicator of litter wetness) and whether the sample was a first, second or final depopulation (high-throughput processors tend to depopulate sheds only once or twice).

This study harnessed the efforts of researchers and industry activity to identify on-farm factors that significantly influenced the numbers of *Campylobacter* on post chill carcass neck skins and in broiler house litter. However, to confirm and investigate further the mechanisms that are operating, additional experimental work is required. Some of the additional studies could be undertaken at small expense, by making use of existing standard operations within some companies. For example, one UK processor sexes birds and holds different genders in different houses. Therefore, the consequences of gender on *Campylobacter* colonisation could readily be investigated further using birds farmed by that company.

KEY OVERVIEW OF THE FINDINGS OF THIS STUDY

- Discussions with the UK broiler processing industry revealed a strong industry preference for the use of three pooled neck skins as a single test sample to determine the degree of carcass contamination by campylobacters.
- Previous surveillance had used a combination sample composed of neck and breast skin.
- When the two sample types were compared, the pooled neck skins were found to be significantly more heavily contaminated with campylobacters compared with breast skin.
- Consequently, a change of test sample to pooled neck skin would make it more difficult to achieve any contamination reduction target set from previous surveillance.

- Information relating to the farm rearing of chicken broilers and the slaughter and dressing of chicken carcasses was collected from the UK processors.
- Matched to the collected growing and processing information was a quantitative test result of campylobacters in either broiler house litter and/or from five sets of three pooled neck skins from each flock processed.
- The quality of the donated *Campylobacter* test result was assessed by proficiency testing. Test results from ineffectively-performing laboratories were not used for this study.
- In combination; the farm, process and laboratory testing information was used to construct models that attempted to predict *Campylobacter* numbers initially on carcasses.
- The initial model revealed that on-farm factors were 3.5x more important than processing plant factors in influencing the numbers of campylobacters on post-chill broiler neck skins.

- In the slaughterhouse, the initial model identified the chilling, washing and de-feathering process stages as being statistically-significantly correlated with the numbers of campylobacters on carcasses

- Further investigations were targeted towards the on-farm growing of birds because changes there would have the most impact in reducing carcass contamination.

- For processor-owned integrated farms supplying high-throughput slaughterhouses a second model was constructed. The factors which increased numbers of campylobacters in litter were identified to be

    - Bird age, with other birds more likely to be colonised
    - Allowing dogs into broiler houses
    - The presence of darkling beetles (which are an indirect indicator of litter wetness)
    - Whether the Broiler shed had been previously depopulated

- For independently-owned farms supplying lower-throughput slaughterhouses a third model revealed the significant predictors of numbers of *Campylobacter* in house litter were
    - Bird gender
    - Age at slaughter
    - Shed size
    - Shed construction material

- Modelling is useful in identifying what exerts influence on *Campylobacter* contamination but it does not provide information on the mechanisms operating that cause any influence. The significant factors identified are discussed in the report in the context of previously-published literature. Some of the identified risk factors require experimental work to determine the nature of the influence.

TABLE OF CONTENTS

EXECUTIVE SUMMARY **3**

KEY OVERVIEW OF THE FINDINGS OF THIS STUDY **6**

TABLE OF CONTENTS **8**

TABLE OF FIGURES **10**

TABLE OF TABLES **11**

A COMPARISON OF *CAMPYLOBACTER* NUMBERS RECOVERED FROM TWO DIFFERENT CHICKEN BROILER-DERIVED SAMPLE TYPES. **13**

- BACKGROUND AND JUSTIFICATION FOR A COMPARISON OF TWO DIFFERENT CHICKEN BROILER-DERIVED SAMPLE TYPES 13
- MATERIALS AND METHODS 15
- RESULTS AND DISCUSSION 19

PROFICIENCY TESTING AS A METHOD FOR THE QUALITY ASSURANCE OF INDUSTRY-DONATED *CAMPYLOBACTER* TEST RESULTS **29**

- INTRODUCTION 29
- MATERIALS AND METHODS 30
- RESULTS AND CONCLUSIONS 35
  - *Proficiency trial summaries* *36*
    - Trial one 36
    - Trial two 37
    - Trial three 44
    - Trial four 45
    - Trial five 45
    - Trial six 46
    - Trial seven 47
    - Trial eight 48
  - *A summary of the proficiency testing scheme* *50*
- **APPENDIX 1 A GENERAL HISTORICAL BACKGROUND DESCRIBING A NEED FOR PROFICIENCY TESTING** 52
- **APPENDIX 2** AN EXAMPLE OF A NOTIFICATION OF AN UPCOMING TRIAL 54
  - *Proficiency tests for enumeration of campylobacters for laboratories testing in the context of the joint government/industry working group on Campylobacter.* *54*
    - Outline protocol for examination of liquid samples from poultry neck skins 54
    - Reference 55
- **APPENDIX 3** FORMS A AND B FOR THE REPORTING OF RAW PLATE COUNTS AND REPORTABLE VALUES RESPECTIVELY 56
  - *FORM A: Enumeration of campylobacters for laboratories examining poultry-derived samples in the context of the joint government-industry working group on Campylobacter* *56*
- **APPENDIX 4** A SELECTION OF REPORTS RETURNED TO LABORATORIES AFTER PROFICIENCY TESTING. 61
  - *An example of a performance report from trial one. (Participant report of the FSA proficiency test for the enumeration of campylobacters for laboratories testing in the context of the joint government-industry working group on Campylobacter. February 2010).* *61*
  - *An example of a performance report From trial two* *65*
- **APPENDIX 5** THE RAW PLATE COUNTS SENT TO THE PARTICIPATING LABORATORIES (WITH ANSWERS PROVIDED SEPARATELY AFTER A RETURN HAD BEEN MADE) 70

MULTIVARIATE STATISTICAL ANALYSIS OF COLLECTED INFORMATION TO DETERMINE WHAT PROCESSING PRACTICES INFLUENCE BIRD COLONISATION ON FARM AND CARCASS CONTAMINATION BY CAMPYLOBACTERS DURING PROCESSING. **71**

INTRODUCTION 71
MATERIALS AND METHODS 73

RESULTS AND DISCUSSION 79

DATA MANIPULATIONS PRIOR TO ANALYSES 79
RESTRICTIONS OF THE STATISTICAL PROCESS 80
A POISSON MODEL IDENTIFYING RISK FACTORS FOR THE COLONISATION OF BROILER CHICKENS ON INDEPENDENT FARMS 86
A POISSON MODEL TO DETERMINE RISK FACTORS FOR BIRD COLONISATION ON INTEGRATED FARMS 89
GENERAL CONCLUSIONS FROM THE MODELS 92
APPENDIX 6 THE QUESTIONNAIRES MATCHED WITH TEST RESULTS USED FOR RISK FACTOR IDENTIFICATION 94
The farm infrastructure questionnaire 94
The flock questionnaire 95
The plant infrastructure questionnaire 98
The plant environment conditions during the processing of a slaughter batch questionnaire 106
The plant environment conditions during the processing day questionnaire 111
APPENDIX 7 THE SQL USED TO CREATE THE TABLES USED TO STORE THE COLLECTED SUPPLEMENTARY INFORMATION AND HARVEST THE COLLECTED DATA 115
APPENDIX 8 FARM CATEGORIES USED FOR THE INDEPENDENT FARMS SUPPLYING LOWER-THROUGHPUT PROCESSORS 118

REFERENCES 119

TABLE OF FIGURES

Figure 1 Typical real-time summary reports of the industry-provided test results made available to the UK poultry processors and research collaborators. 18

Figure 2 A Bland and Altman (1986) plot showing the relationship between the mean of and the difference between individual and paired-neck-and-neck-extension skin samples excised from chicken broilers after the chilling stage of processing. The solid horizontal line shows the average difference between the two types of measure and the lightly dashed lines the 'limits of agreement' (equal to the mean difference ± 2 SDs). 23

Figure 3 Annual percentile summaries of the numbers of campylobacters isolated from post-chill chicken broiler neck skins on 23 UK processing lines representing more than 95% of the national throughput. Summaries are for January to December 2016 (A, n=2417), 2015 (B, n=15100), 2014 (C, n=8265), 2013 (D, n=5684), 2012 (E, n=5279), 2011 (F, n=3291). Test results generated by laboratories with unsatisfactory proficiencies were excluded from the summaries 27

Figure 4 Changes in the numbers of contaminated neck skins in each of the three hygiene bands agreed by the Joint Working Group by year. 28

Figure 5 An example flowchart of key dates relating to a typical proficiency test 34

Figure 6 A summary of the test results reported for the lenticule examinations 63

Figure 7 A summary of the test results reported for the suspension examinations 63

Figure 8 Anonymised Z-score distributions for FSA *Campylobacter* proficiency test two and the six samples which contained campylobacters. For samples 2 and 7, there are Z-scores which became more than two only after the outliers were removed. 69

Figure 9 Dynamic control placement was used to create customised data entry forms on a farm-by-farm basis to make the site easy-to-use for users. In the figure above, the form for the shed detail data was dynamically-created in response to a previous question that asked 'How many sheds are there on the farm?'. 75

Figure 10 The results of an initial run of a Poisson regression within a multilevel framework 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. 81

Figure 11 A visual depiction of the differences between the counts predicted by the model shown in **Figure 10** and the measured numbers of campylobacters on chicken neck skins. 82

Figure 12 A Poisson regression undertaken within a multi-level framework describing the sources of variance influencing carcass *Campylobacter* load in UK slaughterhouses. Parameter estimates are in green with their standard errors shown within the brackets. 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 slaughterhouses and farms respectively and depict the respective impact

of each term to the observed variance. Sin and Cos are transformations of TofYear (the sequential day number) mapped to 360 degrees. 83

Figure 13 A chart showing the residual difference between the slaughterhouse operations model shown in **Figure 12** and the measured numbers of campylobacters on chicken neck skins. 84

Figure 14 The final model for independent farms produced from the MLwiN multilevel analysis. Parameter estimates are in green with their standard errors shown within the brackets. 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 farms, broiler houses and farm batches respectively and depict the respective impact of each term to the observed variance. Farm categories ($NewFmCat_x$) are listed in **Appendix 8**. 86

Figure 15 An example of the use of the equation shown in **Figure 14**. Here the calculation shows the predicted (mean) log10 *Campylobacter* count for a flock of age 40 days for mixed gender birds, in a metal framed broiler house. 87

Figure 16 The final model produced for integrated farms by the MLwiN multilevel analysis. Parameter estimates are in green with their standard errors shown within the brackets. 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 broiler houses respectively and depict the respective impact of each term to the observed variance. The model calculated the terms exerting significant influence on the $\log_{10}$ *Campylobacter* count. The constant (β_0) in the equation is tied to the first category of each categorical variable and to a bird age of zero. 90

TABLE OF TABLES

Table 1 Scope objectives contributed to by this section of the study 15

Table 2 The relationship between a calculated $\log_{10}$ mean of three individual chicken broiler neck skin *Campylobacter* examinations and the test result of the same three samples physically combined into a single sample. The results were analysed by paired t-test and the difference and standard error (SE) of the difference between samples is reported. The SD reported is the standard deviation of the log mean count of the individual and combination neck skin samples. 20

Table 3 The numbers of campylobacters counted on chicken broiler neck extension skin compared with neck skin taken from the same carcass. SD is the standard deviation and SE is the standard error of the difference between results. 22

Table 4 A summary of *Campylobacter* test results from researcher-collected samples of chicken broiler skin and the results of t-tests for significant differences. Mean log numbers of campylobacters are shown ± the standard deviation. 24

Table 5 A summary of *Campylobacter* test results for researcher-collected chicken skin samples. Test results were sorted into the contamination ranges used for monitoring progress towards the UK *Campylobacter* reduction target for poultry meat. 25

Table 6 A summary of progress towards the *Campylobacter* reduction target in the UK between 2011 and 2016. Pooled neck skin samples were tested to determine the numbers of campylobacters by 23 UK poultry processors. Additional information was added to the originally reported dataset in October 2017. 26

Table 7 Scope objectives delivered by this section of the study. 30

Table 8 The numbers of participating laboratories for each round of proficiency testing 36

Table 9 A summary of the abilities of participating laboratories in converting raw plate counts at various dilutions to reportable numbers of campylobacters using the criteria outlined in ISO 7218 and ISO 10272-2. A correct calculation is denoted ✓ and an incorrect as X. 41

Table 10 Ranking of mean sample Z-scores for participating laboratories (except Numbers 1, 13, 15 and 16). 64

Table 11 Specific comments regarding a laboratory: 64

Table 12 Measurement of repeatability for samples used for the second round of proficiency testing. RSD is the repeatability standard deviation of ten examinations of randomly selected samples from a batch under identical conditions within a short (1-2 h) time interval. A dash (-) denotes a sample which did not contain campylobacters. 65

Table 13 Sample specific rankings (Laboratory 8) 67

Table 14 The scope objectives delivered by this section of the report 72

Table 15 Selected example data from a single FBO that entered a single farm CPH number for farms with different postcodes (yellow highlight) and different CPH numbers for a farm with the same postcode (green highlight). 80

A COMPARISON OF *CAMPYLOBACTER* NUMBERS RECOVERED FROM TWO DIFFERENT CHICKEN BROILER-DERIVED SAMPLE TYPES.

BACKGROUND AND JUSTIFICATION FOR A COMPARISON OF TWO DIFFERENT CHICKEN BROILER-DERIVED SAMPLE TYPES

In 2008, the European Union (EU) undertook a survey of campylobacters in chicken broiler meat in 26 member-states, Norway and Switzerland (Anonymous, 2011), which showed that the UK produced more broilers than any other country in the EU. However, the UK was ranked tenth worst in terms of *Campylobacter* prevalence of broiler carcasses with around 75% of samples testing positive. Around 67% of the samples had contamination of more than 10 colony forming units (cfu) campylobacters/g skin sample. The findings of the EU survey broadly agreed with an earlier report by Adak *et al.* (2005) who estimated that over 250,000 cases of campylobacteriosis and that several dozen deaths were caused annually in England and Wales as a likely consequence of the campylobacters associated with poultry meat.

In response to the EU survey findings, a working group was established with members drawn from UK government branches (e.g. the Food Standards Agency (FSA) and the Department of Rural Affairs (defra)), the British Poultry Council (BPC; a trade poultry processer association), poultry processors, retailers and the British Retail Consortium (BRC; a trade association for larger retailers). The primary purpose of the working group was to identify and implement interventions aimed at reducing the numbers of campylobacters on British poultry meat. In addition, the group pledged to undertake continuous monitoring of processed broiler samples post chill in BPC-member slaughterhouses as one way of monitoring progress towards a reduction target agreed with the Food Standards Agency. Three bands for *Campylobacter* numbers (lowest <100, medium 100-1000 and highest >1000 cfu/g) were set, along with reductions to the percentages of the test samples which fell within each banding. The target for the highest band was a reduction from 27% of samples tested to 10% by the end 2015.

The not insignificant costs of the slaughterhouse-based continuous monitoring programme were met by the UK poultry processing industry who donated the results of examinations of three pooled poultry neck skin samples taken from five sets of carcasses. The testing methodology was based on ISO-10272-2 (2006) and

plants collected test samples on a weekly basis. The test sample was chosen because it is widely used for statutory *Salmonella* testing of poultry carcasses in the EU under the auspices of EC regulation 2075 (2003).

For the EU campylobacters survey, the testing protocol was also based on ISO-10272-2. However, the sample collected for microbiological testing comprised a piece of neck skin and the skin from between the neck and breast region (called the neck extension region) of a single chicken carcass. Thus, a significant barrier to using the EU survey results as a baseline, and measuring changes against it, was the use of a different test sample. Consequently, this study reports the findings of a statistical comparison of *Campylobacter* enumerated on both sample types collected from the same flock, with a view to determining the differences in the results consequent on the two sample types.

A secondary consideration when using donated test results was that the testing had been undertaken in a number of testing laboratories rather than the single testing facility per country used for the EU survey. Previously, we have reported that the uncertainty associated with the ISO-10272-2 test method can be significant (Hutchison *et al*., 2006) when identical samples were analysed in just two different laboratories. To determine how consistently campylobacters were enumerated in the participating testing laboratories, a ring trial that involved the testing of subsamples from a homogenous preparation were undertaken. We report the results of a series of proficiency tests of identical samples tested on the same day in different test laboratories.

Finally, after removing the test results generated by outlying laboratories, we report the progress of the British Poultry Industry over a six-year period from 2011 to 2016 towards reducing the numbers of campylobacters associated with poultry meat. This section of the report is concerned with the delivery of the project scope objectives listed in **Table 1**.

Table 1 Scope objectives contributed to by this section of the study

Objective Number	Objective Description
01	Collection of UK poultry processing industry *Campylobacter* test results data and an assessment of the implications of change of sample
03	The creation of systems to summarise and report captured information (with appropriate identity safeguards) to make it available to partner projects, industry representatives and Agency staff
06	Extended collection of UK poultry processing industry *Campylobacter* test results (without making a significant contribution to the collection of matched farm and processing information)

MATERIALS AND METHODS

Sample collection. Excision-based sample collection was undertaken on moving lines during normal commercial processing immediately after the chilling phase of processing. For neck skins, sampling involved turning a sterile 304mm x 177mm stomacher bag (Seward, Thetford, England) inside out over a gloved hand and excising a carcass neck skin to provide a sample mass of at least 10g without removing the carcass from the processing line. Neck and neck extension skin samples were obtained from a single carcass which was typically removed from the processing line to allow sampling. The neck and neck extension skin samples were stored in separate stomacher bags each labelled with a unique carcass identifier. All skin samples were excised using sterile scissors and stored in the stomacher bags on crushed ice until the commencement of microbiological examination.

Microbiological examination. Maximum recovery diluent (MRD, Oxoid, Basingstoke, UK) was added to each sample (9:1, w/w) before homogenisation for 1 minute using a stomacher (Model number BA 6021, Seward, UK). Volumes of diluent were removed from the sample for quantitative determination of campylobacters directly, or for mixing with other samples to form a combination sample. In both cases, the removed volumes

were vortex mixed (Genie, Fountain Valley CA USA; vortex mixer 2) for 10s to ensure a homogenous distribution of bacteria. *Campylobacter* were enumerated using the ISO-10272-2 protocol (International Organization for Standardization 2006). All decimal dilutions were made using MRD and plating was onto modified charcoal cefoperazone desoxycholate agar (mCCDA, Oxoid). Incubation was under microaerobic conditions (CampyGen sachets, Oxoid) at 41.5°C for 48h. Confirmation of *Campylobacter* spp. was by phase contrast microscopic examination of five colonies picked from Columbia blood agar subculture to confirm corkscrew motility, in addition to a lack of visible growth at 25°C under microaerobic conditions and at 41.5°C under aerobic incubation after 48h. In addition, presumptive colonies were confirmed by oxidase activity and an inability to ferment lactose and sucrose.

Collection and reporting of UK poultry processing industry test results. A relational database (SQL Server 2008, Microsoft Corp. Redmond WA. USA) was used to store the microbiological test results. Three methods for the collection of industry test results were used. Copies of laboratory test result certificates were collected from participating plants. The laboratory test method was checked to ensure compliance with the ISO-10272-2 reference method, and the data were entered into the database by manually retyping. Data entry errors were identified by periodic double entry of a portion (~10%) of the test results and comparison of the two datasets. An inputting error of 1% (1 error in 100 entered fields) triggered retyping of all data from an entire session by a different person and subsequent re-comparison of both data sets. Results were also collected as electronic documents (e.g. spreadsheets (MS Excel 2010; Microsoft) or comma-separated value files) directly from testing laboratories. Electronic result submissions were electronically transformed (Excel) if required (i.e., to convert test results into a standard reporting format of cfu/g) and directly pasted into the database. All transformations were independently checked to ensure electronic submission was free from data manipulation errors. Three slaughterhouses entered their own test results into the database using a web interface; an approach that was not widely adopted. Basic validation of dates, bacterial numbers, and sample types that were entered into the web database was undertaken to ensure sensible and appropriate inputs and to prevent the introduction of malicious computer code designed to disrupt the database. No independent verification of test results was undertaken for web-based results entry.

Processors were provided with an anonymised identity code that was used for all reporting. Results were reported in a manner agreed with the poultry processors supplying the test results. A range of reports were

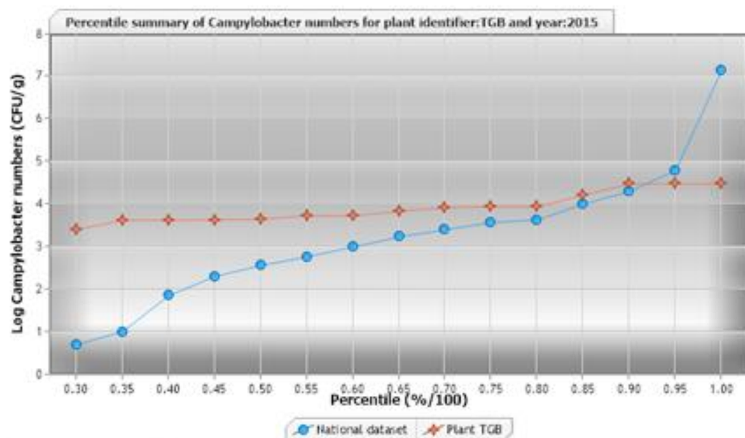
constructed, which included summaries for individual plants, a comparison between individual plants and the national dataset that contained all participating plants and group summaries for larger processors with multiple line and processing plants. Results reporting was also made available to research collaborators after authorisation by the FSA project officer and to comply with Scope objective number three. Examples of typical reports that were created and available in real time are presented below as **Figure 1**.

Statistical Analyses. The bacterial numbers counted from neck extension skin or neck skin samples obtained from the same carcass were compared using the methodology of Bland and Altman (1986). In brief, the range between the two sample types was compared by evaluating bias, assessed as the mean log difference between the two sets of counts and ± twice the standard deviation of the differences, and bias tested using a paired t-test. Chi Squared or Fisher's exact tests were used as appropriate to test for any significant differences in the distribution of test results grouped as scores derived from counts into histogram-style bins. For all tests, the threshold for significance was $P<0.05$ unless otherwise stated.

Select the required timeframe and Approvals Identifier and then click the button.

Approvals Identifier: TGB Timeframe: 2015 Go

Data Source	No. Campy counts >0 and <100 (% total)	No. Campy counts 100-1000 (% total)	No. Campy counts >1000 (% total)
Plant Identifier: TGB	91 (36.546%)	76 (30.522%)	82 (32.931%)



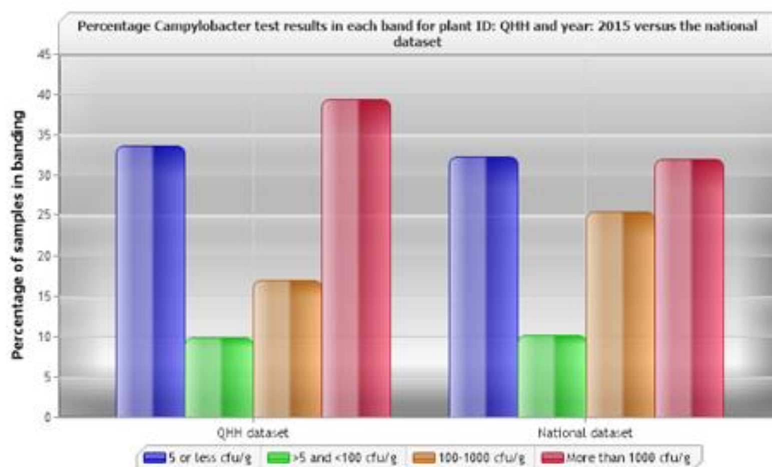
Go to the main menu

Please Note:
We round the data to three decimal places so the percentages may not add up to exactly 100%.
The NaN constant is returned when the result of an operation is undefined e.g. when dividing 0/0.

Select the required timeframe and Approvals Identifier and then click the button.

Approvals Identifier: QHH Timeframe: 2015 Go

Data Source	% counts 5 or less|	% counts more than 5 and less than 100 |	% counts between 100-1000 |	% counts more than 1000
QHH	33.668	9.924	16.959	39.447
All plants	32.342	10.191	25.49	31.976



Go to the main menu

Please Note:
We round the data to three decimal places so the percentages may not add up to exactly 100%.
The NaN constant is returned when the result of an operation is undefined e.g. when dividing 0/0.
NaN will normally only appear as a result if a plant has not reported (m)any results.

Figure 1 Typical real-time summary reports of the industry-provided test results made available to the UK poultry processors and research collaborators.

RESULTS AND DISCUSSION

Table 2 shows the results when the calculated log10 mean of three randomly-selected, independently-tested chicken neck skin samples were compared with the log result of the same three samples physically combined into a single test sample.

Statistical comparison by t-test showed that there were no significant differences between the calculated mean and physically-combined results. The observation was sustained across samples collected from four individual plants and also when the results for all four plants were compared *en bloc*. When the comparison was treated as two different methods for measuring the same parameter (Bland and Altman 1986), there was also no meaningful difference between the two sets of measurements. These findings mean that it is possible to test chicken skin samples individually for *Campylobacter* numbers and use those results to calculate what the test results of a physically-combined test would have been. A similar result has been shown previously for swab samples taken from red meat carcasses (Hutchison et al., 2005); although we believe this is the first time a similar finding has been reported for excised chicken skin. The finding is important for this study because, using the general approach, it is possible to compare the campylobacters associated with different skin sample combinations to circumvent the fact that traditional microbiology does not allow samples to be tested more than once.

Table 2 The relationship between a calculated log_{10} mean of three individual chicken broiler neck skin *Campylobacter* examinations and the test result of the same three samples physically combined into a single sample. The results were analysed by paired t-test and the difference and standard error (SE) of the difference between samples is reported. The SD reported is the standard deviation of the log mean count of the individual and combination neck skin samples.

Plant identifier	Mean log mean count of batches of three randomly-selected neck skins tested individually (cfu/g) ± SD	Number of results (samples tested)	Mean log *Campylobacter* numbers of the same three samples physically combined into a single sample (cfu/g) ± SD	Number of samples	P value (paired t-test)	Difference between physically-combined and calculated mean (cfu/g)	SE (cfu/g)
A	3.45 ± 0.31	10 (30)	3.46 ± 0.45	10	0.960	0.01	0.11
B	3.28 ± 0.46	10 (30)	3.31 ± 0.40	10	0.903	0.03	0.21
C	3.33 ± 0.75	10 (30)	3.37 ± 0.80	10	0.432	0.04	0.05
D	4.07 ± 0.61	10 (30)	4.10 ± 0.72	10	0.557	0.03	0.05
Combined	3.53 ± 0.62	40 (120)	3.56 ± 0.68	40	0.676	0.03	0.06

Table 3 shows how the numbers of campylobacters on the neck extension skin compared with the neck skin sample type when both sample types were excised from the same carcass. The lower counts of approximately 1 log for the neck extension skin (**Table 3**) were significant (paired t-test, P <0.05) for all four plants where samples were collected and also when the test results from all four plants was compared *en bloc*. **Figure 2** is a Bland and Altman plot showing the mean of the paired counts against their differences. There was a marked positive slope in the relationship between mean and difference (P = 0.35), however, this was strongly driven by the two points on the right-hand side of the plot and significance was lost when they were removed (P = 0.77), meaning it is only safe to assume a constant offset when converting from one measure to the other. The finely dashed lines in **Figure 2** shows the 95% 'limits of agreement' (the mean difference ± 2 x SD of the mean difference) between the two measures as being from -0.44 to 2.27 log cfu/g, that is the variability that could be expected when converting from one measure to another. Given such poor limits of agreement it is obviously not practicable or useful to convert between two individual measurements. However, if multiple measures are taken then the 0.92 difference does provide a useful guide to the difference that would be expected overall between the two different types of measure.

Table 3 The numbers of campylobacters counted on chicken broiler neck extension skin compared with neck skin taken from the same carcass. SD is the standard deviation and SE is the standard error of the difference between results.

Plant identifier	Mean difference between neck skin and neck extension skin test result ± 2SD of the difference (cfu/g)	Number of samples compared	SE of the difference between sample types	P value of comparison (paired t-Test) between neck skin and neck extension skin counts
A	1.14 ± 1.24	20	0.14	1.13×10^{-7}
B	0.77 ± 0.94	20	0.11	6.02×10^{-7}
C	0.91 ± 1.92	20	0.22	5.45×10^{-4}
D	0.84 ± 1.04	20	0.12	7.38×10^{-7}
Combined	0.92 ± 1.36	80	0.08	1.3×10^{-19}

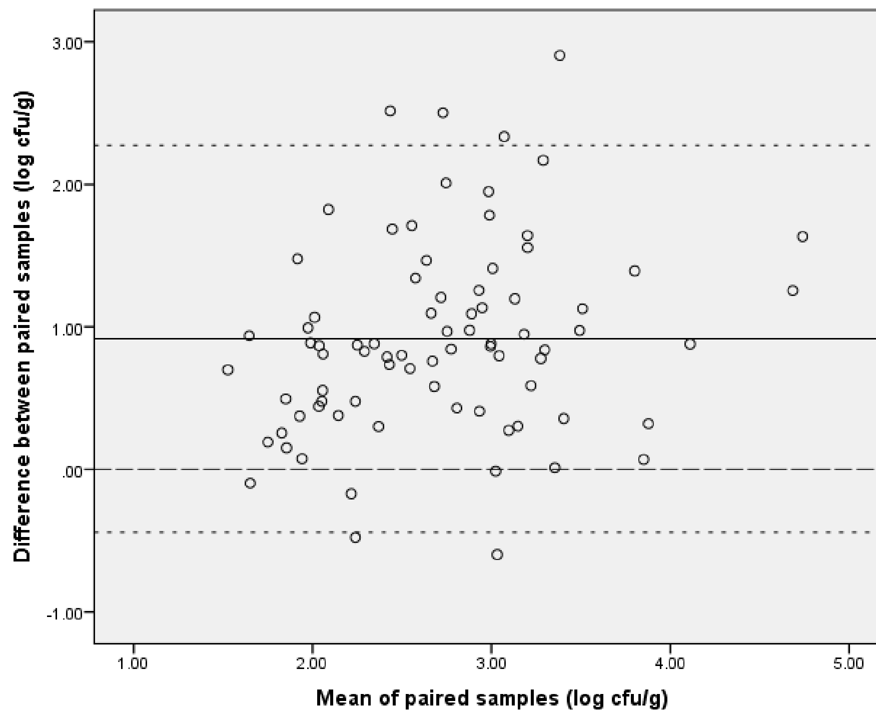


Figure 2 A Bland and Altman (1986) plot showing the relationship between the mean of and the difference between individual and paired-neck-and-neck-extension skin samples excised from chicken broilers after the chilling stage of processing. The solid horizontal line shows the average difference between the two types of measure and the lightly dashed lines the 'limits of agreement' (equal to the mean difference ± 2 SDs).

The calculated approach was used to further investigate the real-world implications of using the EU surveillance data as a baseline to measure progress towards the reduction target taking into consideration the higher counts on neck skin compared with neck extension skin. The studies used the results of individually-tested sample components and a calculated combined result. The comparisons were the calculated combined neck and neck extension skin taken from the same carcass compared with the same neck skin combined with two neck skins taken from near-adjacent birds on the processing line. The results (**Table 4**) show that for three out of the four plants where samples were collected, the counts from the two sample types were significantly different. For the remaining plant, P was 0.05; which was on the cusp of significance. When the results from all four plants were analysed *en bloc*, the two sample types were strongly significantly different. It was more cost-effective for the UK poultry processing industry to take one sample for statutory *Salmonella* testing and to use that same sample for the voluntary *Campylobacter* testing. However, changing

the skin type tested from a combination of neck and neck extension skin to pooled neck skin would make it more difficult to achieve the UK target for *Campylobacter* reductions on broiler carcasses if compared with the EU surveillance as a baseline, due to the greater numbers on the latter samples.

Table 4 A summary of *Campylobacter* test results from researcher-collected samples of chicken broiler skin and the results of t-tests for significant differences. Mean log numbers of campylobacters are shown ± the standard deviation.

Plant identifier	Mean counts of batches of three randomly-selected neck skins tested individually ± SD		Mean counts of neck skin and neck skin extension tested individually ± SD		P value
	Mean log mean *Campylobacter* numbers (cfu/g) ± SD	Number of results (samples tested)	Mean log *Campylobacter* numbers (cfu/g) ± SD	Number of samples	(t-Test)
A	3.45 ± 0.31	10 (30)	3.05 ± 0.54	20	0.050
B	3.28 ± 0.46	10 (30)	2.78 ± 0.73	20	0.045
C	3.33 ± 0.75	10 (30)	2.60 ± 0.82	20	0.022
D	4.07 ± 0.61	10 (30)	3.45 ± 0.71	20	0.027
Combined	3.53 ± 0.62	40 (120)	2.97 ± 0.76	80	<0.001

Table 5 contains the information required to allow an assessment of how much of an impact the sample change would cause. The table sorted researcher-collected and tested results into the histogram-style bins that were used to determine if the *Campylobacter* reduction target for poultry meat had been met. Based on the test results of samples collected in four high-throughput chicken processing plants, it was apparent that the three pooled neck skin sample had higher numbers of test results in the highest banding compared with the sample type used for the baseline survey.

Table 5 A summary of *Campylobacter* test results for researcher-collected chicken skin samples. Test results were sorted into the contamination ranges used for monitoring progress towards the UK *Campylobacter* reduction target for poultry meat.

Skin sample type (number of samples)	Number of samples in each banding (percentage %)		
	<100 cfu g^{-1}	100-1000 cfu g^{-1}	>1000 cfu g^{-1}
Single neck extension (80)	32.00 (40.00)	37.00 (46.25)	11.00 (13.75)
Single neck (120)	80.00 (6.67)	36.00 (30.00)	76.00 (63.33)
Neck and neck extension (80)	9.00 (11.25)	31.00 (38.75)	40.00 (50)
Three pooled neck (40)	0.00 (0.00)	6.00 (15)	34.00 (85)

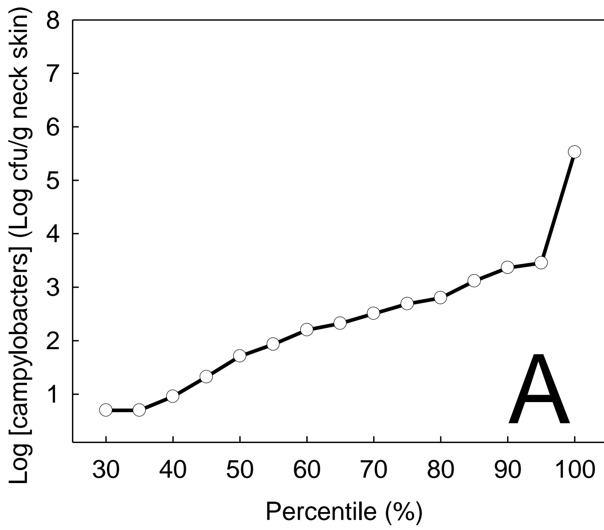
An exact Chi square test using the UK baseline survey bin values as the expected range showed the differences in sample numbers assigned to each bin were significantly elevated (P<0.001) for the pooled neck skins compared with the EU baseline survey sample type.

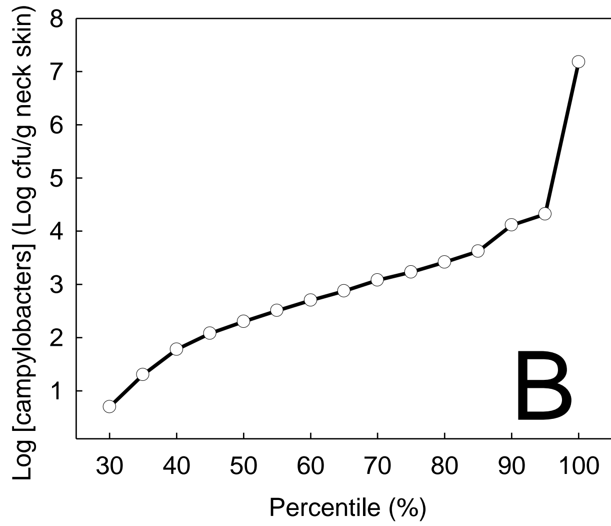
Progress towards the 2015 *Campylobacter* reduction target was also assessed over an almost six-year period using test results donated by the UK poultry processing industries. An anonymous, percentile-based overview of the distribution of the donated test results by year is shown as **Figure 3** and progress towards the performance target is shown as **Table 6**. The shapes of the graphs shown in **Figure 3** between the 30th and 95th percentiles were similar across all six years. However, for years 2015 and 2014, there were small numbers of exceptionally highly contaminated neck skins which contained more than 7 log cfu/g neck skin. In general, the majority of neck skin samples were contaminated below 4 log cfu/g. The results depicted in **Figure 3** and **Table 6** are from a survey representing more than 95% of the UK national throughput over a period of several years. A Chi Square test of the table as a whole showed a highly significant difference between banding between years. In particular, the 2015 dataset had elevated numbers of neck skins in the most contaminated band and reduced numbers of neck skins in the least contaminated band compared with the other years (**Table 6**). Thus, using industry-supplied test results, and a dataset of around 15,000 results for 2015, the JWG reduction target that the highest level of contamination, (>1,000 cfu/g neck skin) would fall to 10% by the end 2015 was

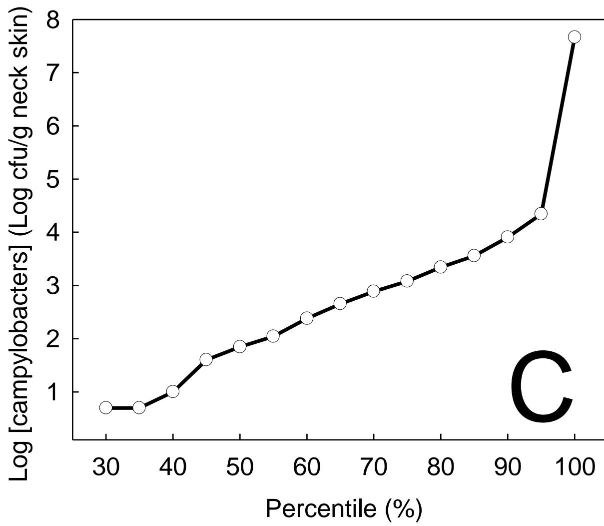
not met. However, the least contaminated chickens (<100 cfu per gram), did not get any worse than the measured baseline of around 42% (**Figure 4**).

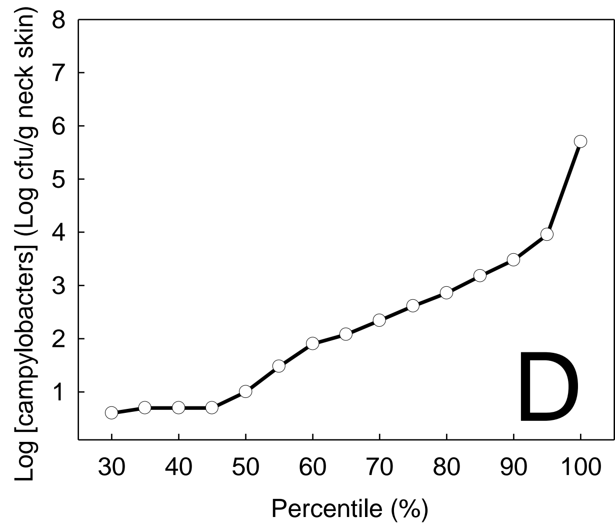
Table 6 A summary of progress towards the *Campylobacter* reduction target in the UK between 2011 and 2016. Pooled neck skin samples were tested to determine the numbers of campylobacters by 23 UK poultry processors. Additional information was added to the originally reported dataset in October 2017.

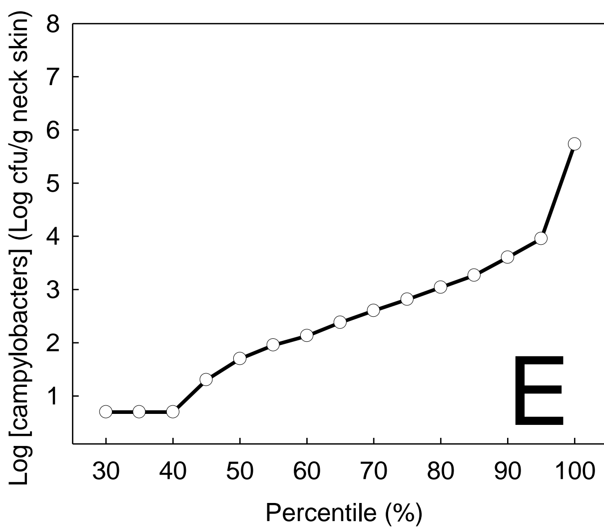
Year	Percentage (%) of samples in each band (number of samples in each band)		
	<100 cfu/g	100-1000 cfu/g	>1000 cfu/g
Jan-Dec 2016	54.60 (1577)	28.13 (785)	17.27 (550)
Jan-Dec 2015	42.53 (6636)	25.49 (3799)	31.98 (4989)
Jan-Dec 2014	50.54 (4165)	22.53 (1857)	26.93 (2219)
Jan-Dec 2013	60.56 (3548)	21.97 (1287)	17.48 (1024)
Jan-Dec 2012	54.37 (2887)	24.14 (1282)	21.49 (1141)
Jan-Dec 2011	60.31 (1969)	22.82 (745)	16.88 (551)











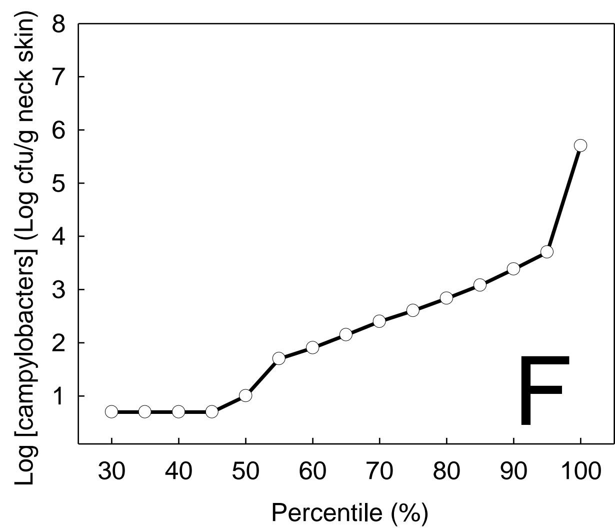


Figure 3 Annual percentile summaries of the numbers of campylobacters isolated from post-chill chicken broiler neck skins on 23 UK processing lines representing more than 95% of the national throughput. Summaries are for January to December 2016 (A, n=2417), 2015 (B, n=15100), 2014 (C, n=8265), 2013 (D, n=5684), 2012 (E, n=5279), 2011 (F, n=3291). Test results generated by laboratories with unsatisfactory proficiencies were excluded from the summaries

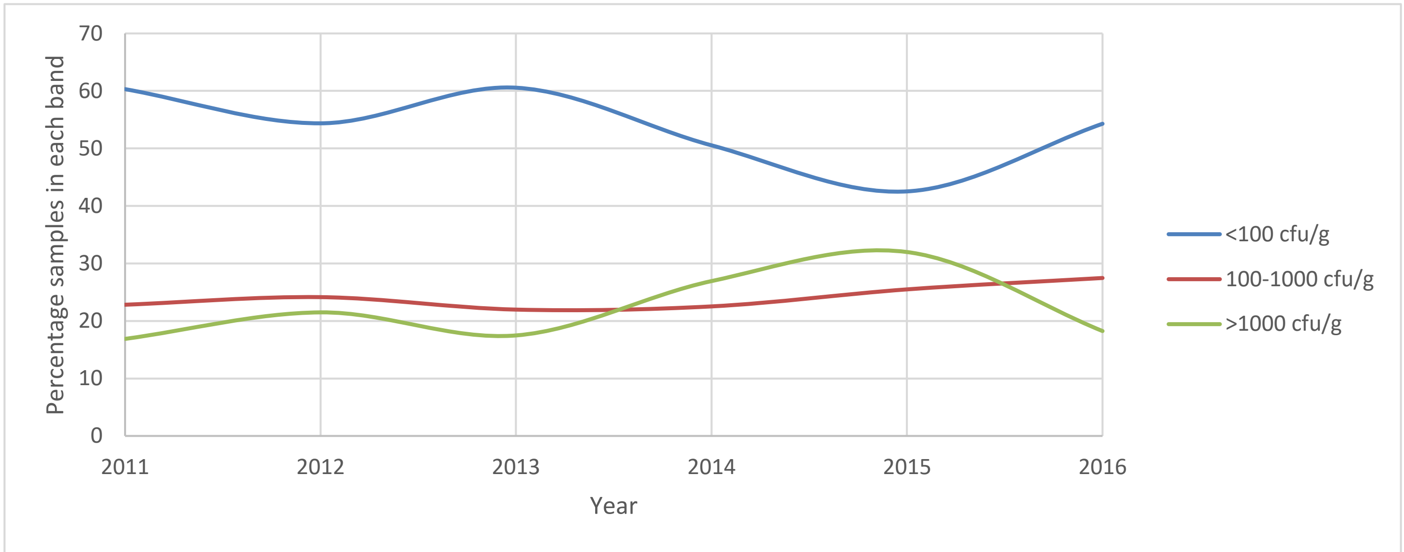


Figure 4 Changes in the numbers of contaminated neck skins in each of the three hygiene bands agreed by the Joint Working Group by year.

PROFICIENCY TESTING AS A METHOD FOR THE QUALITY ASSURANCE OF INDUSTRY-DONATED *CAMPYLOBACTER* TEST RESULTS

INTRODUCTION

In 2001 the FSA initiated surveillance on campylobacters and commissioned a UK wide survey of retail chicken, undertaken from April to June 2001, to determine the prevalences of *Salmonella* and *Campylobacter*. Three laboratories analysed the samples with Scottish samples (n=794) analysed at one laboratory, Northern Irish samples at another (n=797) and English and Welsh samples (n=3,275) at the third. The results (Anon. 2003) showed the respective prevalences of *Campylobacter* to be 75.4%, 76.7% and 45.4%. Contemporaneous surveys of retail chickens running around the same time found *Campylobacter* prevalences of 83% in chickens in England (Jørgensen et al. 2002), and 71% in Wales (Meldrum et al. 2004). The disparity in campylobacter prevalences between surveys running at similar times and different testing laboratories suggested that in the FSA survey, the laboratory attempting to isolate campylobacters from English and Welsh samples may have had significant deficiencies.

Subsequently the FSA strategic plan for 2005-2010 set the aim: ‘We will work with industry to achieve a 50% reduction in the incidence of UK-produced chickens which test positive for *Campylobacter* by the end of December 2010’. However, the baseline set was that found in the problematic survey of 2001, noted above. A second FSA survey to determine the prevalence of *Campylobacter* in retail chicken in the UK was undertaken in 2007 and 2008 (n=3,274). During the study, a subset of samples (n=927) was analysed by both enumeration and enrichment and combining both methods the prevalence for the subset was 65.2%. However, the enrichment method used showed a prevalence of only 33.9% for all samples, and for the subset it had a false negative rate of 62% when compared with the corresponding quantitative result. A similar survey undertaken in Northern Ireland, incubating samples at both 37°C and 42°C, found a prevalence of 91% (n=336) (Moran et al. 2009). Additionally, in 2008, the European Union (EU) undertook a survey of broiler carcasses to determine the prevalence of *Campylobacter*, and the UK prevalence was 86% (Anon. 2010). Thus, the enrichment

methodology used in the FSA survey may have been less than optimal for the detection of poultry-associated campylobacters.

This section of the study had a primary aim to provide appropriate quality-assured test materials for all laboratories undertaking FSA-funded research relating that enumerated campylobacters in poultry. Where laboratories were found not be operating proficiently, the intent was that the project team would proactively assist laboratories to ensure appropriate standards were met on all FSA related studies and help prevent issues of the type described above.

This section of the study contributes to the project scope objectives listed as **Table 7**.

Table 7 Scope objectives delivered by this section of the study.

Objective Number	Objective Description
04	Undertake assessments of the *Campylobacter* testing results and the provision of assistance to atypically-performing laboratories
08	Extended proficiency testing of laboratories that test for campylobacters

MATERIALS AND METHODS

Preparation and management of contact details. Initially, participation in proficiency testing was made a compulsory requirement for those processors who had agreed to supply chicken skin test results for incorporation into the database. As the PT scheme expanded, organisations such as universities and government agencies, who were undertaking *Campylobacter* testing for research or surveillance purposes on behalf of the FSA, were also subject to mandatory participation. For the participating processing plants, a request was made through the Joint Working Group (JWG) that at least one processing plant-based contact be

nominated and their phone number and an email address provided. Although those details were the minimum requirement, what was typically provided by plants were the contact names and details for the plant technical manager, a laboratory contact, a senior plant manager, the corresponding details for a retailer supplied by the plant and an FSA-nominated contact (typically the original FSA project manager). Also included were the address details of the processing plant and the testing laboratory (if different). For the universities and government agencies, contact details were for a FSA-nominated contact, a laboratory manager and a senior manager within the organisation.

Systems were put in place to handle logistical and administrative exceptions and anomalies. For example, a number of processors did not operate their own labs and used external contract laboratories. The same contract laboratory was occasionally used by two or more different processors. Similarly, several processors supplied the same retailer and so there was duplication of retailer contacts. Some contract testing laboratories were located at more than one site, but one senior laboratory contact was provided for all the sites. The contact lists were used to notify the processors, researchers, retailers and laboratories of upcoming PTs. The laboratory address details were used to courier the test samples to the labs. The logistical and administrative systems installed ensured that each contact with duplicated details received notifications of upcoming trials only once; that each testing lab received only one set of test samples; that each retailer contact received the result reports for all of processing plants that supplied their stores.

Contact details were held in a relational database (MS-SQL server) and were changed throughout the duration of the PT scheme as appropriate. The main basis of prevention of duplication was use of the SQL SELECT DISTINCT command to retrieve a single instance of contact information.

Notification of trials. Typically, notifications and results were sent electronically using mail merge to populate email addresses and unique form fields such as contact names, processing plants and other details. Over the course of the PT scheme several standardised documents for notification of an upcoming trial were created. These documents were sent not less than one month before the sample arrival date and included a notification letter (**Appendix 1**), a flow chart showing key dates (**Figure 5**), an initially detailed protocol that was reduced to outline over time (at the request of the JWG), and two forms (A and B; **Appendix 3**) for the provision of reported results and the raw counts used to calculate the reported results, respectively.

Sample preparation. For trial one, four of the samples were lyophilised lenticules containing *Campylobacter*, purchased and quality-assured by PHE, Colindale. For all the other PTs, samples were microbiological suspensions prepared in-house at the University of Bristol. To ensure that test samples had an authentic background micro-flora, all test samples (even those from un-colonised birds) were derived from UK broiler neck skins collected post-chill in a range of different slaughterhouses. Samples were typically collected when members of the project team were visiting plants for other purposes. Two types of flocks were targeted; final clearance flocks, which served as a source of positive samples whilst first clearance flocks were regarded as likely sources of samples containing no or low numbers of campylobacters. To prepare PT samples without campylobacters, neck skins from first-clearance flocks were frozen and thawed at least twice. Neck skins for the generation of samples containing campylobacters were stored refrigerated (2-3°C) for not more than 48h before the commencement of analysis. Such storage does not cause significant reductions to numbers of campylobacters on broiler neck skins (Hutchison et al., 2006a).

To ensure samples had a homogeneous distribution of campylobacters, neck skins were stomached for 5 minutes in maximum recovery diluent (MRD; Oxoid, Basingstoke, UK) (1:9) and the diluent filtered through a 0.45 μm nitrocellulose filter (Sartorius, Epsom, UK) to remove fat and other gross detritus. The resulting MRD containing campylobacters was decanted into sterile universals and the headspace gassed using a custom-formulated mixture of 8% (v/v) carbon dioxide, 7% (v/v) oxygen, and 85% (v/v) nitrogen (British Oxygen Company, Guildford, UK). Vials were vortexed for 2 minutes and gently agitated during the removal of 200μl aliquots into pre-labelled sterile bijoux bottles. The headspace in the bijoux bottles was also replaced with a microaerophilic gas mixture. The samples were shipped in refrigerated biobottles to laboratories for next day delivery, before noon.

Testing to determine homogeneity of campylobacters between samples. The homogeneity of the test samples was checked by a single researcher within the food safety microbiology group in the School of Veterinary Sciences at the University of Bristol. Ten replicates of each of the six samples that were typically used for a PT were tested. The samples were selected randomly from the pool of prepared materials prior to dispatch to the participating laboratories. The relative repeatability standard deviation (RSD) was calculated.

Sample homogeneity was considered to be acceptable if it was below the 52% variance limitations of colony counting generally as quoted by ISO 10272-2:2006 (Cowell and Morisett 1969). The repeatability values for the multiple sample examinations were provided to participants as part of the test report.

Calculations, outliers and extremes and ranking by absolute Z score determination. In keeping with the IHPPTA protocol, proficiencies were calculated as follows. Calculations were performed using the raw plate counts reported by laboratories on Form A to determine if calculation errors had been made. Although there is no correct method for this conversion, the protocol described by section 10 of ISO 10272:2 (2006) was used as a reference to identify gross mistakes giving rise to order of magnitude errors, such as an incorrect handling of dilution factors. For those samples where errors were made, laboratories were contacted and asked to explain how the results had been worked out. The error was noted on their PT report and the corrected result was used for subsequent analyses.

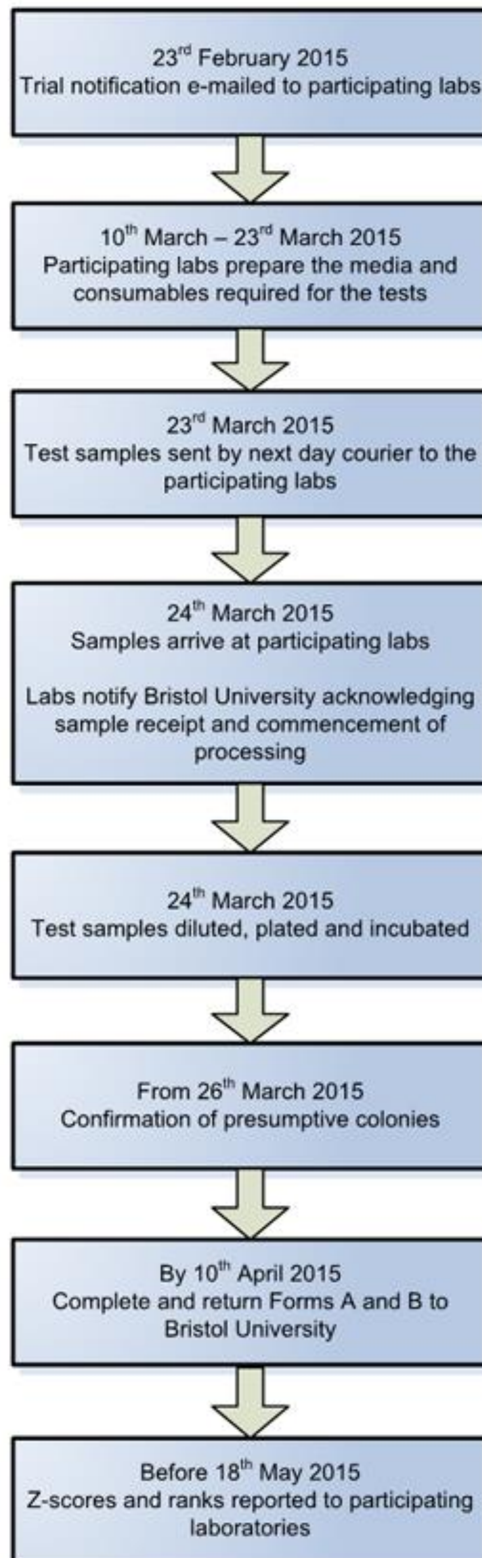


Figure 5 An example flowchart of key dates relating to a typical proficiency test

All results were log_{10} transformed prior to calculation. A group geometric mean value and standard deviation was calculated for each sample from all of the results submitted by all participating labs. The standard deviation was assigned a value of Z=1 and results reported as more than Z=2 away from the group mean were termed outliers. Results more than Z=3 away from the group mean were termed extremes. Outliers and extremes were removed from the group dataset and the labs that had submitted the results were ranked jointly last for the samples in question. After removal of the outliers and extremes, the geometric mean value and standard deviation were recalculated. Laboratories were then ranked by their results, using the absolute distance from the group mean in terms of Z.

RESULTS AND CONCLUSIONS

Eight rounds of PT were undertaken as part of this scheme (including the first two rounds that were merged into a single PT). The numbers of participating laboratories differed between trials as laboratories joined or left the scheme in response to the end of research projects, the sale of laboratories into larger groups with re-organisation, and repeated poor PT performance leading to dismissal by the customer. The labs were in the UK as well as in continental Europe. A summary of the numbers of participating labs for each trial and the trial testing dates is shown on **Table 8.** On average there were more than 20 participating laboratories for each PT. Several laboratories subdivided their samples and had multiple technicians undertake testing. Although every lab was provided with a detailed individual report for every technician during this study, for every trial, this resulted in more than 300 collated reports overall. Therefore, only a selection of randomly-chosen reports is provided (**Appendix 4**).

Table 8 The numbers of participating laboratories for each round of proficiency testing

Proficiency test number	Number of participating laboratories	Sample testing date
1	16	07/11/2011
2	23	26/04/2012
3	26	08/09/2012
4	23	08/05/2013
5	22	02/10/2013
6	20	05/02/2014
7	26	22/10/2014
8	28	24/03/2015

PROFICIENCY TRIAL SUMMARIES

TRIAL ONE

A main finding from the initial trail was that many laboratories might welcome guidance on the conversion of raw plate counts into the values used to populate laboratory reports.

Trial one was undertaken in November 2011. Of the 16 participating laboratories only 13 returned meaningful test results. The temperature of the test bottles on receipt of samples by the laboratories ranged from 4° to 14°C. Statistical analyses showed that these different temperatures had not significantly influenced the numbers of campylobacters in the test samples. Checks on each laboratory's spreadsheet of results showed that several laboratories had difficulties with the basic task of calculating correctly bacterial numbers of campylobacters per lenticule or per ml neck-skin sample from raw plate counts. In addition, two laboratories reported that campylobacters were present in samples from a campylobacter-negative flock and one laboratory reported campylobacters in a PHE-certified, sealed, sterile control lenticule. Each laboratory was ranked by its mean Z-score, (also commonly referred to as a standard score) allowing performance comparisons between labs to be made. It was noted that a small number of laboratories had subdivided their

samples and made multiple returns for different technicians. In the test reports, it was stressed that the results of a single initial PT should be interpreted cautiously, since they were a snapshot of how a single technician (or a small number of technicians) performed over a short period of time.

All the participating laboratories were offered free tuition in the application of the ISO 10272-2:2006 protocol, the identification of different *Campylobacter* colony morphologies on CCDA plates and the biochemical confirmation of presumptive campylobacters. The worst-performing laboratories were most aggressively targeted. During a training day, six participating laboratories attended, along with the FSA project officer. However, none of the worst-performing labs participated in the day's training, which took place in February 2012.

TRIAL TWO

In summary, trial two also revealed that some laboratories would benefit from guidance in the conversion of raw plate counts to reportable numbers. Consequently, some tutorial materials for the calculations and answers were prepared and made available to any participating laboratories that requested them. As a response to queries from PT1, a variety of approaches were tried for laboratories that reported no detections in samples with small numbers of campylobacters.

Trial two made some attempt to address feedback made by the JWG on PT one. In brief, there were a small number of poorly-performing laboratories in PT one. A significant amount of effort was expended by the researchers addressing multiple rounds of queries from multiple labs aimed at finding flaws in the trial approach or justify their performance. Whilst these queries were tolerated, the effort stopped short of providing the raw datasets for recalculation (on the grounds it was sensitive, although anonymous). One common query from PT one was how non-detections of campylobacters in samples containing low numbers of campylobacters were handled.

In general, a variety of similar approaches are commonly used to allow log transformations of microbiological test results below the limit of detection (LoD) of a test method. These included the substitution of half the LoD for counts below the test method sensitivity (Hutchison et al., 2007), which was the method used by PT one. In

addition, the replacement of below-LoD values with 1 (Jackson et al., 2000;Roberts et al., 1980); the addition of a low count of <1 such as 0.9 (Bauermeister et al., 2008) or 0.1 (Russell and Axtell 2005) or lower are also common in the literature. More involved methods of solving the issue include the addition of 1 to zero values before log transformation followed by subtraction of the same amount after the transformation (Saathoff et al., 2004). Overall, these solutions can all be summarised as variations on the general strategy of substitution of a low value for a result below the LoD of the test method. For PT two, the results were calculated using the three simplest approaches and the findings were prepared.

A low value (e.g. 0.000001 cfu/ml) substitution log transformed to -6. When calculating Z-scores, it is the distance from the mean of all test results that is important. For example, if the mean test result for all the laboratories was 50 cfu/ml (unlogged) and one laboratory recorded a result of 28 cfu/ml, the result's distance from the group mean was -22 cfu/ml. Conversely, a result of 85 cfu/ml would be 35 cfu/ml from the mean. Since it is the absolute distance from the mean which is important for Z-scores, the minus sign is removed from all results below the average. For that reason, the 0.000001 log transformation to -6 becomes 6 during the Z-score calculation. In PT two, 6 logs were equivalent to 2-3 standard deviations for all the samples containing campylobacters. Since there were six samples which contained countable numbers of campylobacters for this round of testing, when the average Z-score was calculated, the low value substitution resulted in a penalty weighting of between 0.3 and 0.5 for each instance of a non-detection of campylobacters in samples containing the bacterium. A substitution of a very low value had the most impact on the PT two rankings, but the impact of that substitution was quite minimal overall.

The other two alternative methods of allowing log transformation of results (a substitution of 1 or using half LoD) below the detection limit of the method caused very minor differences in the rankings. At the proficient end of the scale, the top three laboratories had their positions re-arranged by both methods. At the least proficient end, three of the bottom four laboratories (labs 9, 11 and 2) had a minor re-arrangement of their ranking. Although overall, most of the participating laboratories which did not report a result below the LoD had their rank improved by two or three places, labs 6, 12 and 17 did not report a below LoD for a sample containing campylobacters and were disadvantaged by up to three ranks because of the substitution to $1x10^{-6}$ cfu/ml. The general improvements for most labs were at the expense of the laboratories who had reported below an LoD for a sample containing measurable numbers of campylobacters. Each with at least a single

report of a below LoD for a sample containing campylobacters, the most affected were labs 3 (reduced by 3 ranks), 5 (reduced by 7 ranks), 8 (reduced by 2 ranks), 9 (reduced by 1 rank), 11 (reduced by 1 rank) and 15 (reduced by 7 ranks; **Table 3**). In general, the range of mean Z-scores tended to be smaller, although not significantly so (paired t-test; P=0.47) when $1x10^{-6}$ was used as the substitute. The sole exception was the least proficient laboratory which did not detect any campylobacters in any samples and thus accumulated six penalty weightings and a mean Z-score of 3.32. The narrower range may have a possible consequence of requiring mean Z-score calculation to four significant figures. Since the rank differences for each of the different strategies was minimal, the original approach of substituting half the LoD of the test method was retained for future trials.

In PT two, we also saw similar issues with the calculations required for the conversion of raw counts to reportable numbers of campylobacters. Therefore, tutorial materials were prepared that were designed to inform on the various rules and special cases specifically described by ISO 10272-2 and more generally by ISO 7218. The general idea was that work sheets (**Appendix 5**) were sent to all participating laboratories to be returned by a deadline. Once a return had been made a series of answers (and workings if required) were sent back to the laboratory. A summary of the laboratories' abilities to convert raw counts to reportable values is shown as **Table 9**.

It was considered very important by the project team and the JWG that laboratories were able to accurately convert raw plate counts to reportable values. Thus, quite detailed feedback on their performance was provided back to the participating laboratories.

Laboratory 2 had grasped the basics of conversion and was, overall, able to correctly calculate straightforward counts. However, the lab did not have an apparent awareness that both ISOs define special cases. Consequently, lab 2 treated plates with excessively high counts of >300 the same as plates with counts >10 and <150. The lab was unable to correctly calculate plates which used a volume that was not 100µl; consistently overestimating the counts for 333µl platings by a factor of x10. The percentage of correct calculations for lab 2 was 38.9%.

Laboratory 4 completed the calculations to a very high standard. Q7 and 14 had minor breaches in how the presentation of >300 cfu on a plate were handled. Like many labs, for Q18 all three counts at the neat dilution

and all three at the x10 dilution were used. Since one of the neat counts was >300 cfu, it should have been excluded. The percentage of correct answers for lab 4 was 83.3%.

Laboratory 7 was difficult to assess. The lab converted all the plate counts to reportable values and provided these calculations. In addition, the lab attempted to apply the various rules and special cases described by the ISO and reported these. The typical answer format was 'a number OR other number calculated after applying a special case'. For a few answers three choices were offered. Most of the time, one of the answers was correct. The two special cases that were missed were Q6 which should have been reported as "Organisms present but less than 4 cfu/ml"; and Q18 where the lab did not use two of the three counts at the neat dilution and all three at the x10 dilution. Credit was given if one of the answers provided was correct; the percentage of correct answers was 88.9%.

Laboratory 8 also showed a basic ability to correctly calculate bacterial numbers from raw counts. However, the lab showed a poor awareness of special cases and used very low and very high counts in their calculations. There was one instance of a rounding error and another where a decimal place was dropped. Laboratory 8 could not calculate a reportable value using two counts from one set of 333μl dilutions and three from another set of 333μl dilutions. The percentage of correct answers for lab 8 was 33.3%.

Laboratory 9 provided a set of comprehensive notes to accompany their answers and employed a 'multiple answers' strategy similar to Lab 7. The email returning the lab's answers was headed 'queries for you to answer!' (sic) and was quite aggressive in tone. This laboratory was disappointed with its performance in the first round of proficiency and there was extended dialog with the lab owner who felt strongly customers should not have been shown the proficiency results. It seemed likely that Lab 9 saw these tests as a challenge to try and catch the research team out rather than an opportunity for assistance. How the PT results were distributed was a matter decided by the FSA project officer and the JWG rather than the research team. Many the points in the notes were of the type "you can't have this as a count because it exceeds the limit of 150 stated in the ISO", when the point of the exercise was to see how the labs would handle such counts if they encountered them on a raw plate.

Table 9 A summary of the abilities of participating laboratories in converting raw plate counts at various dilutions to reportable numbers of campylobacters using the criteria outlined in ISO 7218 and ISO 10272-2. A correct calculation is denoted ✓ and an incorrect as X.

Question number	Laboratory number											Count of wrong answers
	2	4	7	8	9	10	11	14	15	17	18	
1	✓	✓	✓	✓	✓	✓	✓	✓	X	✓	✓	1
2	✓	✓	✓	✓	✓	✓	✓	✓	X	✓	✓	1
3	✓	✓	✓	X	✓	✓	✓	✓	X	✓	✓	2
4	X	✓	✓	X	✓	✓	X	X	X	✓	X	6
5	X	✓	✓	X	✓	✓	X	✓	X	✓	✓	4
6	X	✓	X	X	✓	X	X	✓	X	✓	X	7
7	X	X	✓	X	✓	X	X	X	✓	✓	X	7
8	✓	✓	✓	✓	✓	X	X	✓	X	✓	✓	3
9	X	✓	✓	X	✓	✓	✓	✓	X	✓	✓	3
10	X	✓	✓	X	✓	✓	✓	✓	X	✓	✓	3
11	✓	✓	✓	X	✓	✓	✓	✓	X	✓	✓	2
12	✓	✓	✓	✓	✓	✓	X	✓	X	✓	✓	2
13	✓	✓	✓	✓	✓	X	X	X	X	✓	✓	4
14	X	X	✓	X	✓	✓	X	✓	✓	✓	✓	3
15	X	✓	✓	X	✓	✓	X	X	✓	X	X	6
16	X	✓	✓	✓	✓	✓	✓	✓	X	✓	✓	2
17	X	✓	✓	X	✓	✓	✓	✓	X	✓	✓	2
18	X	X	X	X	X	X	X	X	X	X	X	11
Total correct	**7**	**15**	**16**	**6**	**17**	**13**	**8**	**13**	**3**	**16**	**13**	

For Lab 9, the multiple answer strategy meant that at least one of the options provided was correct. Lab 9 was treated the same way as Lab 7 and credited with a correct answer if one of the options was correct. A minor rounding error in Q18 was the only point of trivial note. The percentage of correct results was 94.4%.

Laboratory 10 also demonstrated proficiency in converting raw counts to numbers. Only minor issues were identified; the laboratory was able to identify when too low or too high counts were encountered, but it did not always follow the ISO advice for special cases. However, the reports all stated the problem with a count and, typically, provided an estimate result.

There were some issues with Laboratory 11. In summary, the lab was able to convert basic raw plate counts to reportable values without issue. However, the lab had only a rudimentary awareness of special cases. The researchers considered it likely the laboratory did not read the relevant sections of both ISO documents. The special cases missed included: use of counts >300 cfu and <4 cfu, an inability to report in the form >150 cfu/ml for high counts and the incorrect selection of the most appropriate dilutions when counts over three orders of magnitude were provided. Like most laboratories; for Q18, laboratory 11 was unable to successfully calculate a reportable value using two counts from one set of 333μl dilutions and three from another set of 333μl dilutions.

Laboratory 14 also provided good evidence they could reliably convert raw plate counts to reportable values. The laboratory manager at Lab 14 was off sick and so the calculations were done by a junior staff member. Minor issues were that for Q4 both dilutions were used for the calculation but only the one <300 cfu should have been used. There was an apparent arithmetic error for Q7 which was incorrect by one order of magnitude. A special case of not using an arithmetic mean was missed for Q13 and not excluding counts of <4 cfu for Q15. Finally, for Q18; the lab did not use two of the three counts at the neat dilution and all three at the x10 dilution. The percentage of correct answers was 72.2%.

Either Laboratory 15 undertook calculations using a method which was different to the ISO method, or they were unable to round numbers to 2 significant figures effectively. Consequently, almost every answer was incorrect. To be fair to the laboratory however, the answers provided were very close in most cases (only one or two decimals difference). The laboratory correctly-handled special cases for Q7, Q14 and Q15. The percentage of correct answers was 16.7%.

Laboratory 17 completed the exercise to a high standard. The only issues of note were for Q15 low counts at the 1/100 dilution were used for the calculation and for Q18, the >310 count was used for the calculation. The percentage of correct answers was 88.9%.

Laboratory 18 had also grasped the basics of conversion; the majority of the answers were either correct or within an acceptable margin as a consequence of rounding. In addition, when rounding to 1 dp (the ISO states 2 significant figures), there were inconsistencies of the type that 1.43 was rounded to 1.5 in one instance and 364 was rounded to 360 in another. The laboratory used counts in excess of 300 colonies and less than 4 colonies for calculations. Where counts were too high for use in calculations and were to be reported as (for example) >3.00 x 108. The lab consistently reported as 50% of the upper limit e.g. > 1.50 x108. The root of that issue is that one ISO says it's fine to use plates with up to 300 colonies whereas the other limits colony numbers to 150 of the target organism. The percentage of correct calculations was 72.2%.

In summary, because of the tutorial for the conversion of raw counts to reportable numbers, it was apparent that almost all of the laboratories (with the exception of Lab 15) had read the relevant sections of each of the ISOs and could convert raw plate counts to reportable values without issue for standard cases. Things become less clear when the counts fell into the ISO-defined special cases. Overall, most laboratories were aware that some of the raw counts were atypical and thus required special handling. Whether the special handling applied was correct (as defined by the ISOs) was quite hit-and-miss.

It was apparent that compared with their performance in the first round of proficiency testing most laboratories had shown improvement in their ability to convert raw plate counts to reportable values. A further set of laboratory-based proficiency testing was scheduled towards the end of April 2012. Prior to the commencement of PT3, labs were provided with an Excel spreadsheet template, which accepted raw plate counts and made appropriate consideration of the ISO special cases to accurately convert raw counts to reportable numbers.

One final consideration for PT2 was that there were complaints made regarding the method used to anonymise participating laboratories. Until PT two, the labs had been sequentially numbered in the (broadly random) order they had signed up to the PT scheme. There were several laboratories undertaking surveillance on behalf of the FSA included in PT two. Although not individually identifiable, these laboratories could be identified as one of the new group by their high sequential number. Therefore, the way in which laboratories

were anonymised was changed after PT2. A three letter alphanumeric was randomly generated and assigned to each participating laboratory.

TRIAL THREE

There was nothing of note revealed by trial three. A small number of laboratories were still struggling with conversion of raw counts to reportable values, and there were a small number of mistakes made at the level of individual technicians. However, there was no widespread issues identified and the research team were hopeful that the PT scheme was beginning to have an impact on testing and driving improvements to the quality of information donated by the UK poultry processing industry.

As trial three was in preparation, several labs requested for more than one set of test samples so that they could make multiple returns from multiple technicians. The requests indicated that the PT trials were perceived to be useful by the participating labs. Multiple samples were provided when requested (rather than increasing sample volume in case that changed conditions, and consequently numbers of campylobacters in the vials).

As before, analyses of each laboratory's testing and reporting practices and their test results showed a range of capabilities. For example, one laboratory produced high outliers (overestimates) for three of the five samples whereas another laboratory produced three low outliers (underestimates). The other laboratories showed no bias in the distribution of any outliers they returned. There was some slippage of the improvements to plate counts to reportable values calculations noted after the tutorial run after PT2. For PT three, two laboratories were asked to clarify how they had calculated numbers of colony-forming units (cfu) of campylobacters per ml from their colony counts. For one of these laboratories, the revised calculations supplied as a response used a recognised (non-ISO) method for the calculations. For the other laboratory, the results from three different technicians had been grouped together but had not been labelled as such. Other issues of minor note related to the responses to questions. In two cases, labs answered confirmation questions in a confusing manner claiming that (for example) 5/5 presumptive colonies grew under aerobic conditions on Columbia Agar at 41.5°C but returning a greater than LoD result. However, despite these small

issues; for the first time, none of the laboratories reported campylobacters to be present in a negative sample (sample 4). In keeping with what was observed for PT two, a number of laboratories had difficulty in measuring *Campylobacter* numbers in a sample that contained low numbers of cells (sample 3; mean test result 1.24 log cfu/ml).

TRIAL FOUR

For PT four, there was increase in returns that were not useable. There were 12 results excluded overall because of reporting a false negative or a high or low outlier or extreme.

One laboratory reported four high outliers, and another reported two outliers which were significantly lower than the participating laboratories average results. One laboratory returned negative Z-scores for all samples which is evidence of consistent under-reporting of *Campylobacter* numbers. Other laboratories showed no bias in the distribution of any outliers and Z-scores that they returned. There was still some slippage with the improvements to plate counts to reportable values calculations noted after PT two. For PT four, four laboratories were asked to clarify how they had calculated numbers of colony-forming units (cfu) of campylobacters per ml from their colony counts. For three of these laboratories, the project team corrected the supplied results. None of the laboratories reported campylobacters to be present in a negative sample (sample 4). In contrast to what was observed for PT three, most laboratories showed an improvement by measuring *Campylobacter* numbers in a sample which contained low numbers of cells (sample 6; mean test result 1.52 log cfu/ml).

TRIAL FIVE

A summary of the findings from trial five were that the majority of the laboratories made robust returns that lacked anything of concern to the project team. The main observation of note was that previous PTs used samples with an upper limit of around 1000 cfu/ml. By accident, some of the broiler neck skins used to generate the samples for PT five were exceptionally highly contaminated with campylobacters. A number of laboratories were caught out by the need to plate dilutions of 10^4 or 10^5 in order accurately report the counts in one sample.

For PT five, there were three results excluded overall as a consequence of reporting a high outlier and one result excluded on the grounds it was a low outlier. In addition, one laboratory reported two high outliers, a false negative and failed to enumerate the atypically high numbers of *Campylobacter* inoculated into sample 4. Investigations revealed that the technician that normally undertook *Campylobacter* testing was not available and the testing had been completed by a different technician. It was not clear whether the replacement technician had ever previously tested for campylobacters, or just not participated in proficiency testing. The disappointing results were largely a consequence of not plating six volumes, each of 333 μl, for the 10-1 dilution. A number of technicians were caught out by the high numbers of campylobacters in sample 4 reported their result as a 'greater than' value. There were four laboratories that returned negative Z-scores for all samples, which is evidence of consistent under-reporting of *Campylobacter* numbers. Other laboratories showed no bias in the distribution of any outliers and Z-scores that they returned. There were no significant issues with the conversion of raw plate counts to reportable numbers of cells by any participating laboratory for this round of testing. None of the laboratories reported campylobacters to be present in the negative samples (samples 2 and 5). In keeping with what was observed for PT4, most laboratories were able to measure *Campylobacter* numbers in the sample that contained low numbers of cells (sample 6; mean test result 5.75 cfu/ml).

TRIAL SIX

As for the previous PT trial (PT five), there were no widespread significant issues were identified with this trial. In addition, there were only minor, isolated instances with the conversion of raw plate counts to reportable numbers of cells by the participating laboratories.

For PT six, twenty-six participating laboratories were sent samples. Of these 26 labs, 23 made valid returns. One laboratory declined to participate in the current or any future tests. The same laboratory had failed to make a return for PT five. A different laboratory could not participate in testing schedule due to the personal

circumstances of the person that was responsible for PT trials. However, one week later, this laboratory examined the samples using a polymerase chain reaction (PCR)-based method, although the results confirmed only the presence or absence of the pathogen in each sample. In the format provided, these returns could not be included in any quantitative analyses. The final laboratory that failed to make a valid return provided their results a considerable interval past the required deadline and reported their results as 'lower than' values, which also couldn't be used for statistical analysis.

A number of labs made returns from multiple technicians. In total 30 valid returns were made and multiple submissions from single labs were treated as separate and independent returns for the purpose of Z-score calculation. One of those laboratories compared two different *Campylobacter* plating media.

Following initial analysis, six results were excluded as a consequence of reporting a high or low outliers. One laboratory reported two high outliers, two low outliers, a false negative and a false positive. There were three laboratories that returned outlying results for sample number three, which contained low numbers of campylobacters. However, the majority of the laboratories were able to accurately measure *Campylobacter* numbers in the samples that contained higher numbers of cells (samples 2, 4 and 6). The laboratory that submitted PCR data reported two false negative results for samples 1 and 3 but correctly confirmed the presence/absence of *Campylobacter* in the remaining samples.

TRIAL SEVEN

There was a relatively long time (8 months) between PT six and PT seven . Possibly, that was the reason for the return of issues with the conversion of raw plate counts to reportable numbers of cells. For trial seven, four participating laboratories experienced difficulties with the calculations.

In more detail, two laboratories reported results that were ten times lower than the correct result, and one lab reported five times the correct value. All of the affected laboratories were contract labs, where staff turnover

can be high. One contract lab re-submitted corrections to their calculations three times before they were correct. For those laboratories that failed to submit corrected calculations, researcher-corrected values were used for the PT calculations and a note was made on the report.

Following initial analysis, sixteen results were excluded because of reporting a high or low outlier. One laboratory reported two high outliers, two low outliers, a false negative and a false positive, a return consistent with guessing the vial contents. There were nine laboratories that returned low outlying results for at least one of the samples, and there were seven high outliers, clustered round a single lab that made multiple returns. Most of the laboratories were able to accurately measure *Campylobacter* numbers in the samples that contained high numbers of cells (samples 1 and 5) and lower numbers (samples 2, 4 and 6). Sample 3 did not contain any campylobacters, but there were three returns from one lab that reported a false positive result. The same laboratory did not detect any campylobacters in sample 1 and reported three instances of a false negative. It is plausible that sample 1 and sample 3 were mixed up at that laboratory. A second laboratory also reported campylobacters in sample 3.

TRIAL EIGHT

As for PT seven, there were issues with the conversion of raw plate counts to reportable numbers of cells by four participating laboratories for this round. The labs were exclusively contract labs.

In summary, two laboratories reported all results ten times lower than the correct result. One lab reported results calculated from decimal dilutions ten times higher than the correct value, although calculations from neat plates were correctly calculated. One lab reported a single result that was x10 too low. For this round, in response to unacknowledged repeated requests for revised calculations, the reported numbers that were submitted were used.

Following initial analysis, ten results were excluded because of reporting a high or low outlier. There was no discernible pattern of laboratories consistently reporting high or low values. Most of the laboratories were able to accurately measure *Campylobacter* numbers in the samples that contained high numbers of cells

(samples 2 and 4) and lower numbers (samples 1, 3 and 5). Sample 6 did not contain any campylobacters.

There were three false positive reports, clustered round a single lab that made multiple returns.

A SUMMARY OF THE PROFICIENCY TESTING SCHEME

Several commonly-encountered themes emerged over the duration of the PT scheme. Although no specific analyses were attempted based on the laboratory type, there was a tendency for government and university laboratories to submit results closer to the group mean compared with the contract laboratories. The basis of the better performance was not clear, although it is possible that in the former laboratories, staff would perhaps have better qualifications and education. In addition, the experience of some members of the project team who have worked in government organisations, commercial labs and university posts is that government organisations are better equipped than other laboratories. Although similarly well-equipped, university laboratories are used by students and so at least some of the equipment found there is damaged and in need of repair.

It is likely that equipment such as air displacement pipettes, which can be set to measure specific volumes are more common in government and university laboratories. Calibrated automatic pipettes are more accurate compared with the generic motorised guns with volumes operator-estimated from disposable pipettes that are prevalent in most larger contract laboratories.

All the contract laboratories and some of the government laboratories participating in the PT scheme were independently accredited for quantitative *Campylobacter* testing by a third party such as UKAS or CLAS (Campden Laboratory Accreditation Scheme). None of the university laboratories were accredited, despite having a relatively proficient performance.

The results of the PT scheme were used as an indicator of the quality of the industry-supplied test results. All the supplied results were from either a company-owned laboratory or a contract laboratory. The criteria for exclusion from the national dataset was set by the JWG. If a laboratory submitted a PT return that contained three or more major errors in a single round, their results were subject to exclusion. Major errors were defined as the reporting of a false positive (i.e. a count for a sample that contained no campylobacters) or false negative (a negative result for a sample containing campylobacters) result. In addition, major errors also

included the submission of outlier or extreme results, and any calculation error that resulted in a reported result that was outside of 0.2x or 5x the corrected result. Over the duration of the study, there were 11 sets of results excluded, five of which came from a single testing laboratory. The periods that exclusions were applied were also set by the JWG as the midway points between the problematic round and the previous and future PT rounds. For example, **Table 8** lists the test dates for PT two, three and four as 26/04/2012, 08/09/2012 and 08/05/2013 respectively. Midway between PT two and three is 02/07/2012 and between three and four is 07/01/2013. The results from a lab that had three or more errors for PT three would be excluded from the national dataset between 02/07/2012 and 07/01/2013.

APPENDIX 1 A GENERAL HISTORICAL BACKGROUND DESCRIBING A NEED FOR PROFICIENCY TESTING

In overview, this study was concerned with the large-scale collection of *Campylobacter* test results generated from the examination of chicken broiler neck skins and supplementary data relating to those microbiological test results. The general project strategy was to determine if the collected supplementary information could predict the numbers of campylobacters in the test samples, thereby identifying factors that influenced the degree of contamination of chicken carcasses. In more detail, the supplementary information was used as the basis of a multivariate statistical model for the prediction of *Campylobacter* numbers associated with neck skin excised from chicken broiler carcasses sampled after the chilling stage of processing.

Before any modelling could be undertaken there were several considerations that were required, which related to both the *Campylobacter* test results and the supplementary information. This section of the study focussed on an important issue relating to the laboratory-derived test results. In the main, this study used *Campylobacter* test results that were donated by the UK processing industry and were generated by a constantly-changing number of different testing laboratories. The testing labs were a diverse group comprising commercial contract laboratories, processor-owned labs located in or near slaughterhouses, government-owned labs and laboratories undertaking commercial testing that were attached to a variety of research organisations. For reliable statistical analyses to be undertaken, there was a requirement that all of the laboratory testing outputs were broadly equivalent. Consequently, as part of this study, a proficiency testing (PT) scheme based on standardised samples was designed and established to allow an assessment to be made of the relative quality of the test results donated by industry. At the time this study commenced, there was no commercial *quantitative* PT scheme for campylobacters available in the UK (although a *qualitative* scheme was operated by Defra under the auspices of the Food Analysis Performance Assessment Scheme [FAPAS]).

Formal quality assurance of laboratory outputs by PT is a relatively-recent innovation, gathering momentum in the late 1960s. Wood et al. (1998) noted that formalised PT emerged in the United States from largely-informal, ad hoc, inter-laboratory testing between related laboratories. Early PT operated with a dual purpose that allowed laboratories to demonstrate their proficiency in undertaking a specific measurement and to certify reference test materials for internal laboratory quality-assurance purposes (Wood et al., 1998). In

modern laboratories, reference material certification and PT have become largely separated, with each having diverged into a separate specialism.

In the UK, PT became commonplace because medical biochemists involved in large clinical trials identified a need for reliable results that were comparable between different hospitals and institutions. As PT evolved globally, standards for the approach and design of trials and the interpretation of reported results emerged. Most recently, a collaboration between the International Organisation for Standards (ISO), the International Union of Pure and Applied Chemistry (IUPAC) and the Association of Analytical Communities (AOAC) has resulted in a set of standard guidance intended for use in the food sector called the International Harmonised Protocol for the Proficiency Testing of Analytical Laboratories (IHPPTA). The IHPPTA protocol has become a widely adopted international standard, mostly because of being endorsed by a number of influential organisations including the Codex Alimentarius Commission, the World Health Organisation (WHO), AOAC International and the European Union (Thompson et al., 2006). Since the late 1990s, there has been a PT participation requirement for all well-regarded laboratory accreditation schemes such as those operated by the United Kingdom Accreditation Service (UKAS).

The IHPPTA protocol is involved; summary overviews of the technical aspects of the protocol run to more than 50 pages (de Albano and ten Caten 2014;Thompson et al., 2006). In addition, there are a number of pre-requisites for running an IHPPTA-compliant PT scheme that extend into areas such as staff organisation and responsibilities, the procurement of all materials used in the preparation of certified standards and document version control. Collectively, the IHPPTA pre-requisites underscore the importance of good quality control for all aspects of a PT scheme that is intended to be run indefinitely, with the long-term spread of the associated scheme establishment costs. Clearly, it is beyond the scope of a research project of a few years' duration, and concerned with a single analytical measurement, to mimic full IHPPTA-compliant scheme establishment. However, for the purposes of this study many of the important IHPPTA scheme requirements such as scheme design by a qualified statistician; audit, review and verification of calculations by independently-operating researchers; verification of sample homogeneity; equipment calibration and formal documentation of all records were adopted. The PT scheme for quantitative estimation of campylobacters in chicken skin-derived samples to determine the relative quality of the test results was run on eight occasions between December 2011 and March 2015.

APPENDIX 2 AN EXAMPLE OF A NOTIFICATION OF AN UPCOMING TRIAL

PROFICIENCY TESTS FOR ENUMERATION OF CAMPYLOBACTERS FOR LABORATORIES TESTING IN THE CONTEXT OF THE JOINT GOVERNMENT/INDUSTRY WORKING GROUP ON *CAMPYLOBACTER*.

Trial five

Each participating laboratory will receive six liquid samples from homogenised poultry neck-skins (without pieces of skin) and a bottle of sterile water labelled "control". The laboratories will be notified on the 6th September 2013 to expect the arrival of the samples on the 2nd October 2013. Laboratories will be asked to examine for numbers of colony-forming units of campylobacters by direct plating and to confirm a proportion of the colonies as *Campylobacter* species. The samples will be sent chilled, to arrive by 12.00 noon on Wednesday 2nd October, and the laboratories will be asked to record the time of arrival and measure the temperature of the bottle containing sterile water as soon as the samples arrive. Test samples should be examined on the day of receipt. Results should be sent by email or post to Monika Tchórzewska (M.Tchorzewska@bristol.ac.uk) to arrive no later than the 25th October 2013. Trial results will be reported to participating labs by the 30th November 2013.

Laboratories should notify Dr Monika Tchórzewska by email (M.Tchorzewska@bristol.ac.uk) or phone (0117 3319129) that their test samples have arrived, stating the temperature in the control bottle, and confirming that they will plate the samples out the same day (if they arrive after 2 pm, plating can be done the following morning).

Laboratories are asked to record all of the information required in the two results forms (A and B). Numbers of *Campylobacter* colonies on all plates should be recorded unless numbers are >150 per plate. Results should be calculated as cfu campylobacters per ml sample. If no campylobacters are detected the laboratory should determine their own limit of detection (x per ml liquid sample) and express the result as "less than x….".

OUTLINE PROTOCOL FOR EXAMINATION OF LIQUID SAMPLES FROM POULTRY NECK SKINS

The samples should be processed following your normal laboratory procedure for counting *Campylobacter* spp.; for example, as described in paragraph 9.0 onwards of the ISO 10272-2 method. Plate out the neat suspension and dilutions to 10^{-2}. Pick up to five colonies per sample and check for identity as *Campylobacter*. Do not determine to species level. Make sure you record the information required in the two results forms (A

and B). Numbers of *Campylobacter* colonies on all plates should be recorded unless numbers are >150 cfu per plate. Record the results on the form provided and calculate the number of cfu *Campylobacter* per ml suspension for each sample after considering the results of any confirmations of presumptive colonies that you undertake.

REFERENCE

ISO 10272-2 (2006) Microbiology of food and animal feeding stuffs — Horizontal method for detection and enumeration of *Campylobacter* spp. — Part 2: Colony count technique. International Standards Organisation, Geneva.

APPENDIX 3 FORMS A AND B FOR THE REPORTING OF RAW PLATE COUNTS AND REPORTABLE VALUES RESPECTIVELY

FORM A: ENUMERATION OF CAMPYLOBACTERS FOR LABORATORIES EXAMINING POULTRY-DERIVED SAMPLES IN THE CONTEXT OF THE JOINT GOVERNMENT-INDUSTRY WORKING GROUP ON CAMPYLOBACTER

Trial xxxx

Lab number, and name of person completing form: ……………………………………

Date of completion of form: …………………….. Signature: ………………………………

1 Date and time of receipt of samples:

2 Temperature of the liquid in bottle labelled ‘control’

3 Were the samples received in good condition?
   a. If not, explain problem:

4 Name and composition of diluent used.

5 Name, manufacturer, code number and lot number of plating medium used.

6 Were the plates bought ready-poured or prepared ‘in house’?

   a. How many plates per dilution were inoculated?
   b. What volume per plate was used?

c. How did you produce a microaerobic atmosphere?

d. What was the time and temperature of incubation of the plates?

7 Date and time (morning or afternoon) when the analysis of samples commenced.

8 Were all samples examined on the same day? If not, explain when they were examined.

9 How many colonies per sample were examined for confirmation purposes?

Suspension 1

Suspension 2

Suspension 3

Suspension 4

Suspension 5

Suspension 6

10 Indicate in the table below whether suspect colonies were seen, and which confirmation tests you performed Use the format: number of confirmed colonies/total number of colonies tested e.g. 3/5

	Suspect colonies seen?	Micro-scopy	Oxidase	Catalase	Aerobic growth at 41.5C	Micro-aerobic growth at 25C	Latex agglutination test	PCR test*	Other commercial test*
Suspension 1									
Suspension 2									
Suspension 3									
Suspension 4									
Suspension 5									
Suspension 6									

*Please give details.

11 Identify, using initials, who: a) set up the plates?

b) read the plates and confirmed the colonies?

12 Please complete the Excel spread sheet (Form B) to show numbers of colonies of *Campylobacter* counted.

13 Were the samples examined more than once? If so, please give details and supply the results for all examinations by returning more than one copy of Forms A and B.

Campylobacter Trial xxxx - FORM B: *Campylobacter* colonies - SUSPENSIONS

Name and number of Laboratory:	
Date samples received:	
Date samples examined:	
Volume plated neat suspension:	
Volume plated other suspensions:	

Please provide the raw (i.e. unconfirmed colonies) plate counts and then the reported numbers of confirmed campylobacters in the table below

		Raw count at dilution (cfu/plate)			
Results	Plate no.	Neat	1 in 10	1 in 100	
SUSPENSION 1	A				
	B				
	C				
SUSPENSION 2	A				
	B				
	C				
SUSPENSION 3	A				
	B				
	C				
SUSPENSION 4	A				
	B				
	C				
SUSPENSION 5	A				
	B				
	C				
SUSPENSION 6	A				
	B				
	C				

cfu confirmed *Campylobacter*
per ml suspension

APPENDIX 4 A SELECTION OF REPORTS RETURNED TO LABORATORIES AFTER PROFICIENCY TESTING.

AN EXAMPLE OF A PERFORMANCE REPORT FROM TRIAL ONE. (PARTICIPANT REPORT OF THE FSA PROFICIENCY TEST FOR THE ENUMERATION OF CAMPYLOBACTERS FOR LABORATORIES TESTING IN THE CONTEXT OF THE JOINT GOVERNMENT-INDUSTRY WORKING GROUP ON *CAMPYLOBACTER.* FEBRUARY 2010).

Sixteen laboratories which either undertook testing for UK slaughterhouses and retailers, or for research purposes, participated in a proficiency test for campylobacters during late November 2011. A laboratory, in the Department of Clinical Veterinary Science, University of Bristol, prepared four pulsified samples from chicken neck-skins for testing. The neckskins were taken from *Campylobacter*-infected and -free flocks collected from slaughterhouses after the chill stage of processing. These samples were dispatched chilled, for next day delivery, together with four samples of lenticules supplied by the HPA containing known numbers of campylobacters. Each participating laboratory received eight samples in total. Laboratories were asked to test the samples on the day that they were delivered to determine numbers of colony-forming units of campylobacters by direct plating using their usual test method. Ten replicates of each of the eight samples were examined by the Bristol Laboratory after simulated chilled transport and on the same day as the samples were examined by the other laboratories.

Laboratory calculations were checked for accuracy and corrected if necessary. Statistical analysis of the corrected test results was undertaken using all of the laboratory-supplied data by log transforming bacterial numbers before the calculation of the mean log and standard deviation of the mean log for each sample. Each laboratory was ranked by its mean Z-score (defined as the distance of the reported result away from the mean of all test results in units of standard deviations) calculated from the sum of its absolute Z-scores for each test sample.

Box and whisker plot summaries of the entire range of test results from all participating laboratories are shown in **Figure 6** for lenticules and **Figure 7** for suspensions. Both figures should be interpreted as follows: the buff-coloured boxes represent the 25th to 75th percentiles for the range of test results reported. The lower tip of the whisker (i.e. the structure which looks like an error bar) is the lowest test result value reported and the highest whisker tip is the highest value reported. The black band running through the box is the median value (50th percentile) of the data. Also displayed on the plots are outlying values (o) and extreme values (*). The extreme and outlying values shown on 1 and 2 were not used for the calculations of mean log test results, the standard deviation of the mean log or the distances of individual labs from the mean for all test samples (Z-scores). Outliers and extremes however were included when calculating the Z-scores for each participating laboratory. The first table is a ranked summary of the average Z-score for all of the test samples for participating laboratories. The second table contains laboratory-specific comments

- Of the 16 participating laboratories, 13 returned meaningful test results.
- The temperature of a test bottle on receipt of samples ranged from 4° to 14°C. Statistical analyses showed that these different temperatures had not significantly influenced the numbers of campylobacters in the test samples.
- Checks on each laboratory's spreadsheet of results showed that many laboratories struggled to calculate correctly bacterial numbers of campylobacters per lenticule or per ml neck-skin sample from raw plate counts.

- In addition, two laboratories reported that campylobacters were present in samples from a *Campylobacter*-negative flock and one laboratory reported campylobacters in a sterile lenticule.
- Each laboratory was ranked by its mean Z-score, allowing performance comparisons between labs to be made. However, the numbers of samples that were prepared and tested were quite low. For that reason, the results of this initial trial should be interpreted cautiously since the initial results are a snapshot of how a single technician (or a small number of technicians) performed over a short period.
- Subsequent planned proficiency tests will allow this initial snapshot of performance to be expanded and create a more balanced overview of the performance of each laboratory over time.

The next proficiency trial is scheduled for April 2012.

As part of the activities of the Joint Industry and Government *Campylobacter* working group, support is available for laboratories who would like to address any issues highlighted by this initial round of proficiency testing. For general queries please contact Dr Mike Hutchison (phone 01934 741115 or email mh@hutchisonscientific.com). For assistance with conversions from raw plate counts to cfu/sample or cfu/ml, or for example spread sheets with simulated colony counts which can be returned for checking, please contact Dr Janet Corry (phone 0117 928 9409 or email janet.corry@bristol.ac.uk).

Mary Howell
Hygiene and Microbiology Division, Food Standards Agency

mary.howell@foodstandards.gsi.gov,uk

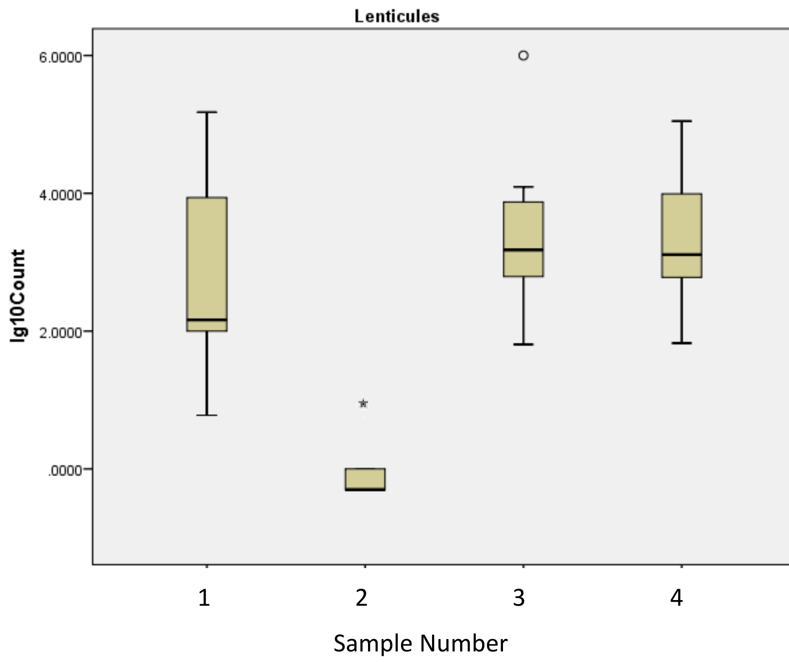


Figure 6 A summary of the test results reported for the lenticule examinations

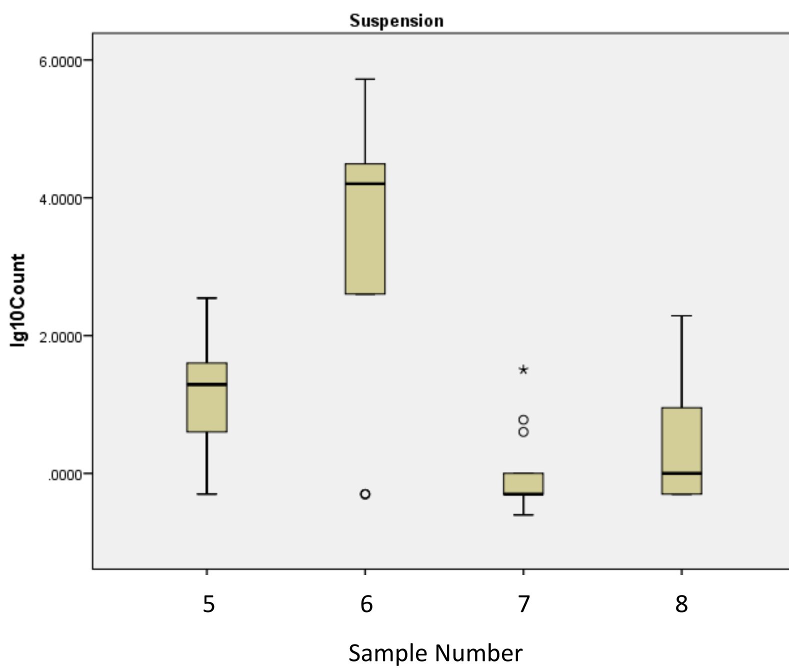


Figure 7 A summary of the test results reported for the suspension examinations

Table 10 Ranking of mean sample Z-scores for participating laboratories (except Numbers 1, 13, 15 and 16).

Laboratory Number	Rank position	Mean Z score (standard deviations)
11	1	0.35
12	2	0.47
7	3	0.49
3	4	0.53
10	5	0.57
14	6	0.61
9	7	0.65
5	8	0.78
17	9	0.83
4	10	1.02
8	11	1.27
2	12	1.56
6	13	1.86

Table 11 Specific comments regarding a laboratory:

Characteristic assessed	Comment
Commencement of sample processing	22nd November 2011
Sample temperature on arrival	5.0°C
Calculation of cfu/lenticule from plate counts	Out by a factor of between x2 and x4 for all three samples reported. Unable to determine the nature of the error
Calculation of cfu/ml from plate counts for suspension samples	Suspension 6 calculated correctly; no other calculations to check
Mean z score	1.27
Rank within participating laboratories	11th out of 13
Result when testing sterile sample	No colonies
Result when testing *Campylobacter* negative sample	No colonies
Outlying or extreme results	None

Trial summary:

For this round of proficiency testing, the number of participating laboratories increased from 16 to 23. As for the previous trial, the participating labs undertook testing for UK poultry producers, poultry processors or retailers, research purposes or food safety investigations purposes. Samples were derived from neckskins of *Campylobacter*-colonised and -free flocks. The skin samples were collected from slaughterhouses after the chill stage of processing. The samples found to be *Campylobacter*-negative were frozen after examination and before use in the trial, in order to further assure that they were negative. Test samples were dispatched by next-day delivery to the participating testing laboratories under refrigerated conditions. Laboratories were asked to examine the samples on the day that they were delivered, and to determine numbers of campylobacters per ml of suspensions using their usual test method. All 23 participating laboratories returned test results. The homogeneity of the samples was checked by Bristol University by examining 10 replicates of each of the eight samples. The repeatability of these multiple examinations are shown as **Table 12**.

Table 12 Measurement of repeatability for samples used for the second round of proficiency testing. RSD is the repeatability standard deviation of ten examinations of randomly selected samples from a batch under identical conditions within a short (1-2 h) time interval. A dash (-) denotes a sample which did not contain campylobacters.

Sample Number	RSD (log cfu/g sample)
1	-
2	0.09
3	-
4	0.10
5	0.09
6	0.16
7	0.08
8	0.06

Laboratories were asked on receipt to measure the temperature in a control bottle of sterile water included with their samples. These temperatures varied from 2.0°C to 10.4°C. Analyses of each laboratory's testing and reporting practices and their test results showed a range of capabilities. One laboratory found no campylobacters in any sample. Dramatic improvement compared to the first trial was observed in the proportion of laboratories which correctly calculated numbers of colony-forming units (cfu) of campylobacters per ml from their colony counts. In addition, only one laboratory reported campylobacters to be present in one sample which did not contain campylobacters. However, a number of laboratories had difficulty in measuring *Campylobacter* numbers in samples containing low numbers.

Statistical analysis of the test results was undertaken using all of the laboratory-supplied data, by converting all cfu detected in *Campylobacter* positive samples to log_{10} before the calculation of the mean log and standard deviation of the mean log for each sample. Each laboratory was ranked by its Z-score for each sample (defined as the distance of the reported result away from the mean of all test results, except the results of the homogeneity test, in units of standard deviations). The rankings for your laboratory are given in **Table 13** together with the mean z score and mean log cfu/ml for each sample.

As for the first trials, although the statistical analysis allowed ranking and comparisons between labs to be made, the numbers of samples that were prepared and tested were quite low. For that reason, the results of these trials should also be interpreted cautiously since the results datasets are still small enough to be considered a snapshot of how a single technician (or a small number of technicians) performed over a short period of time. Subsequent proficiency tests may allow this initial snapshot of performance to be expanded and create a more balanced overview of each laboratory's abilities over time.

Table 13 Sample specific rankings (Laboratory 8)

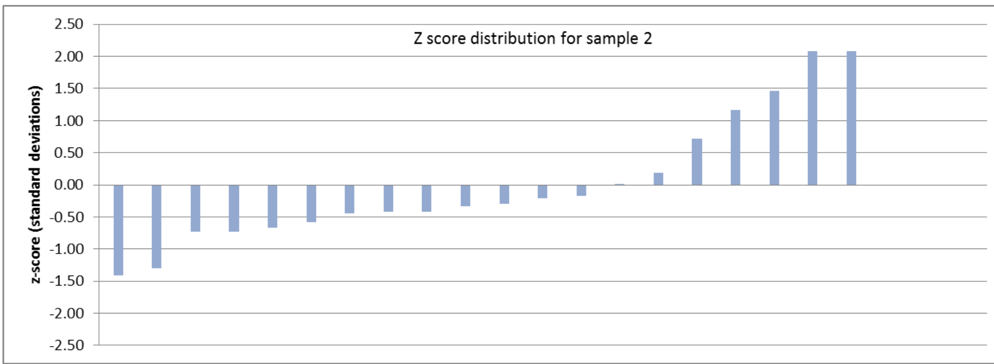
Sample Number	Ranking (/23 participating laboratories)	Absolute Z-score	Mean absolute Z-score for all test labs	Mean log test result for all test labs (log cfu/ml)
1	Correctly reported no campylobacters	-	-	-
2	21	-	0.77	2.27
3	Correctly reported no campylobacters	-	-	-
4	20	-	0.52	2.40
5	22	-	0.83	1.39
6	4	0.23	0.78	1.12
7	20	1.59	0.79	1.47
8	10	0.83	0.86	1.68

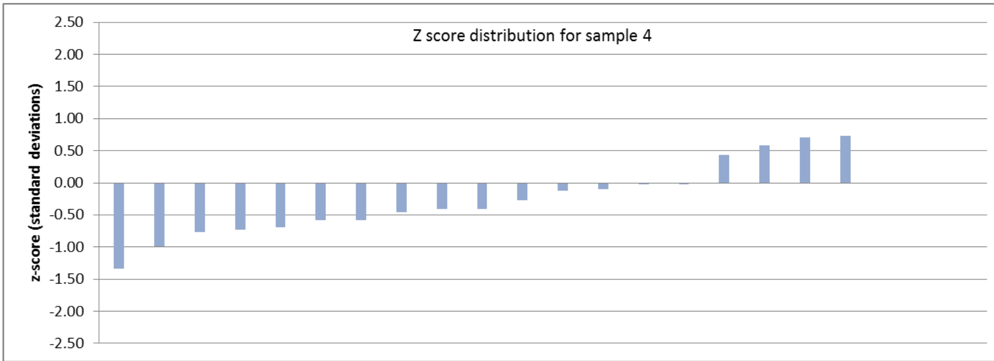
Notes on changes to the method of calculation:

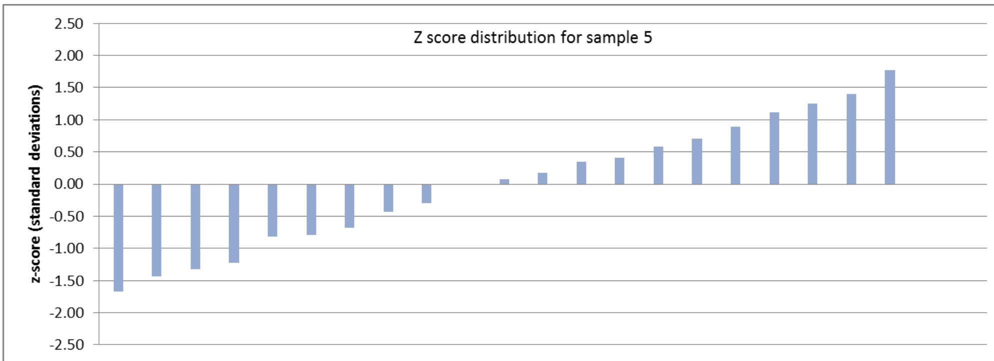
As for trial one, results which were more than two standard deviations from the mean generated from all participating testing labs were excluded on the grounds they were atypical. On occasion, recalculation after the removal of outliers can result in z scores of more than 2 for some laboratories, as was the case for samples 2 and 7.

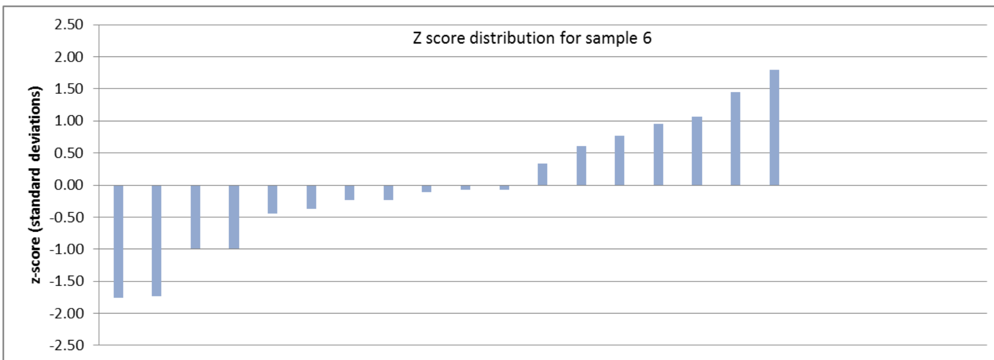
For this round of testing, ranks were not calculated for laboratories where a value below the method limit was reported for a sample which contained campylobacters (i.e. a false negative result). Labs reporting a false negative result were ranked 23rd for that sample (or 22nd equal etc if more than two labs reported a false negative). No overall ranking was calculated for this trial.

An anonymised summary of the distances from the mean are shown as **Figure 8**. The figure has been provided to show how closely clustered some Z-scores were and how ranking alone based on a Z-score may not provide an indication of proficiency relative to other participating laboratories. Each laboratory should look at their Z-score on each of the graphs in **Figure 8** relative to the Z-scores of the other participants to judge their performance


Z score distribution for sample 2
z-score (standard deviations)
2.50
2.00
1.50
1.00
0.50
0.00
-0.50
-1.00
-1.50
-2.00
-2.50


Z score distribution for sample 4
z-score (standard deviations)
2.50
2.00
1.50
1.00
0.50
0.00
-0.50
-1.00
-1.50
-2.00
-2.50


Z score distribution for sample 5
z-score (standard deviations)
2.50
2.00
1.50
1.00
0.50
0.00
-0.50
-1.00
-1.50
-2.00
-2.50


Z score distribution for sample 6
z-score (standard deviations)
2.50
2.00
1.50
1.00
0.50
0.00
-0.50
-1.00
-1.50
-2.00
-2.50

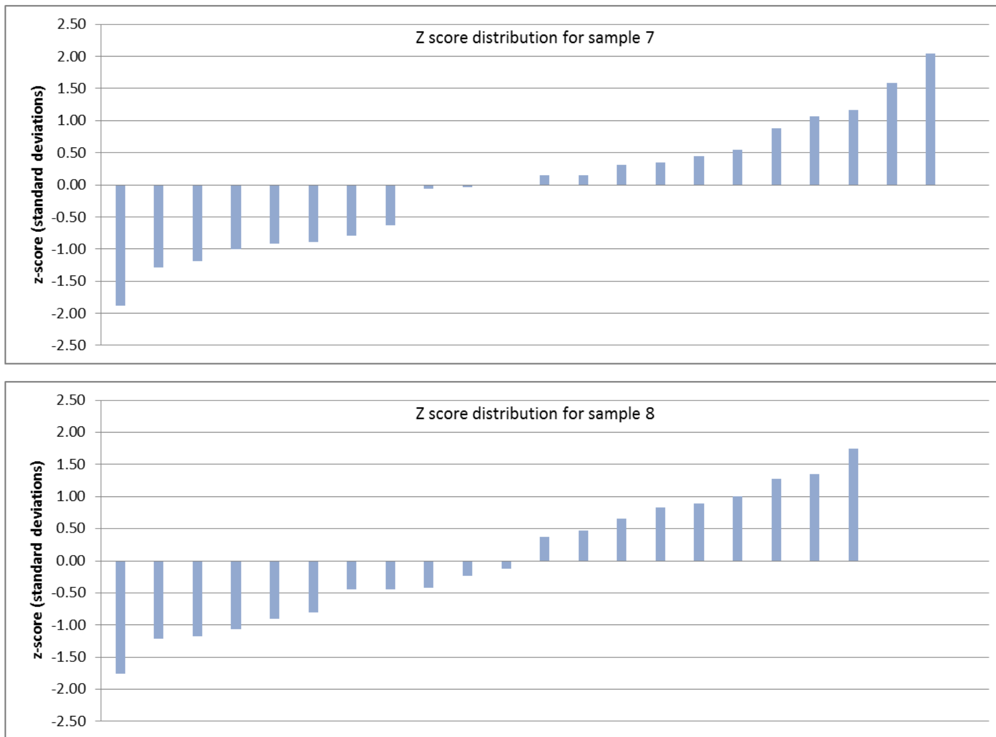


Figure 8 Anonymised Z-score distributions for FSA *Campylobacter* proficiency test two and the six samples which contained campylobacters. For samples 2 and 7, there are Z-scores which became more than two only after the outliers were removed.

Additional laboratory-specific notes

Sample 5 contained small numbers of *Campylobacter*. The mean log result from all test laboratories for sample 5 is shown in **Table 13**.

APPENDIX 5 THE RAW PLATE COUNTS SENT TO THE PARTICIPATING LABORATORIES (WITH ANSWERS PROVIDED SEPARATELY AFTER A RETURN HAD BEEN MADE)

Calculate the number N of microorganisms present per ml of test sample as a weighted mean from two successive dilutions of 100 µl of plated liquid sample. The neat test sample is a liquid.

TMTC is too many to count; ND is not determined

				Dilution				
Question Number	**Neat**	**1/10**	**1/100**	**1/1000**	**1/10000**	**1/10^5**	**Answer**	**Notes**
1	TMTC	TMTC	148	14	0	0	1.47E+05	This is the example from the ISO method EXCEPT we say above we plated 100 µl and the ISO uses 1ml
2	142	15	0	0	0	0	1.43E+03	Straightforword, if you know that neat is a dilution of 1 (10^0)
3	TMTC	TMTC	TMTC	TMTC	89	12	9.18E+06	Really just to see if you can convert between 1/10^4 and 0.0001 or can just type the fraction
4	TMTC	TMTC	340	9	0	0	9.00E+04	Special case: More than 300 at d1 and less than 10 at d2; report estimated count on basis of colonies counted for 10^-3 dilution
5	32	8	0	0	0	0	3.64E+02	Not special case: Less than 10 colonies but more than 4 plus there is a neat count of 32 and so calculate as normal
6	2	0	0	0	0	0	-	Special case: Between 1 and 3 colonies and so should be reported as "Microorganisms are present, but at less than 40 per ml"
7	TMTC	TMTC	TMTC	TMTC	TMTC	335	> 3.00E+08	Special case: more than 300 colonies should be reported as more than 3.00E+08 (if 150 colonies used as the cut-off then >1.50E+08 is also fine)
8	TMTC	TMTC	TMTC	TMTC	TMTC	156	1.56E+08	Special case: Last dilution >10 and <300 so only use last dilution
9	TMTC	145	22	0	0	0	1.52E+04	Nothing special here
10	TMTC	TMTC	140	27	0	0	1.52E+05	Nothing special here

Calculate the number N of microorganisms present per ml of test sample as a weighted mean from dual platings of two successive dilutions of 100 µl of plated liquid sample. The neat test sample is a liquid.

				Dilution										
Question Number	**Neat**	**Neat**	**1/10**	**1/10**	**1/100**	**1/100**	**1/1000**	**1/1000**	**1/10000**	**1/10000**	**1/10^5**	**1/10^5**	**Answer**	**Notes**
11	TMTC	TMTC	128	145	22	14	0	0	0	0	0	0	1.40E+04	Straightforward calculation using scheme specifed by ISO 10272-2
12	TMTC	TMTC	TMTC	TMTC	TMTC	TMTC	149	146	16	13	0	0	1.47E+06	Straightforward calculation using scheme specifed by ISO 10272-2
13	ND	ND	10	13	ND	ND	0	0	ND	ND	0	0	1.15E+03	Special case: two plates containing less than 15 colonies (ISO 10272-2); use arithmetical mean
14	TMTC	TMTC	TMTC	TMTC	TMTC	TMTC	TMTC	TMTC	TMTC	TMTC	320	340	> 3.00E+08	Special case: more than 300 colonies both plates so should be be reported as more than 3.00E+08 (if 150 colonies used as the cut-off then >1.50E+
15	TMTC	TMTC	132	TMTC	21	19	2	1	0	0	0	0	1.43E+04	Should use one plate for 1/10 and both for 1/100; 1/1000 counts are too low

Calculate the number N of microorganisms present per ml of test sample as a weighted mean from triplicate platings of 333 µl of plated liquid sample. The neat test sample is a liquid.

				Dilution				
Question Number	**Neat**	**Neat**	**Neat**	**1/10**	**1/10**	**1/10**	**Answer**	**Notes**
16	145	132	122	12	15	14	4.00E+02	Straightforward calculation
17	55	63	43	4	5	5	1.59E+02	Straightforward calculation - less than 10 colonies but at least 4 so standard case
18	145	140	310	30	20	33	4.80E+02	Don't use the neat dilution with the 310 count; use all three 1/10 dilutions

NB: the answers are presented using a cell format of scientific notation rather than to 2 significant figures, so that those who require it can see the actual calculations (laid out the same way as in the ISOs) that produced the result.

MULTIVARIATE STATISTICAL ANALYSIS OF COLLECTED INFORMATION TO DETERMINE WHAT PROCESSING PRACTICES INFLUENCE BIRD COLONISATION ON FARM AND CARCASS CONTAMINATION BY CAMPYLOBACTERS DURING PROCESSING.

INTRODUCTION

In response to EU-wide surveillance that ranked the UK tenth worst in terms of *Campylobacter* prevalence amongst 26 EU member states plus Norway and Switzerland, a group composed of industry and government representatives was established. The purpose of the group, named the joint working group (JWG), was to reduce the numbers of campylobacters on British poultry meat. In addition, industry pledged to undertake continuous monitoring of processed broiler samples post chill in slaughterhouses as a way of monitoring progress towards an agreed *Campylobacter* reduction in the percentage of chickens that had the highest contamination (>1,000 cfu/g), from the EU-measured baseline of 27% in 2008 to 10% by the end of 2015.

Of particular interest to the JWG was one of the recommendations of the EFSA Baseline B *Campylobacter* in Broilers analyses report (Anonymous 2010b). 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 colonised or contaminated with *Campylobacter* in a country". This report outlines the findings of the FS241051 A (MO1056) study, commissioned as part of a programme by the FSA, aimed at the identification of poultry risk factors for campylobacters on broiler meat in the UK.

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 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 multi-year 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 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 multi-level 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 (Bland and Altman 1986).

This section of the report relates to the scope objectives listed in **Table 14**.

Table 14 The scope objectives delivered by this section of the report

Objective Number	Objective Description
02	The on-going collection of plant characteristics, operations data and layouts
05	Interim multivariate statistical analyses of collected information to determine what processing practices influence carcass contamination by campylobacters
06	Extended collection of UK poultry processing industry *Campylobacter* test results and matched farm and processing information
07	Extended collection of farm and processing conditions information matched with the *Campylobacter* test results
09	Undertake final multivariate statistical analyses of collected information to determine what processing practices influence carcass contamination by campylobacters
10	Report and paper writing

MATERIALS AND METHODS

Test sample sources. FSA project FS241063 (MO1055) was concerned with the characterisation of 23 slaughterhouse process lines to determine any basis in the UK for the EU assertion that some poultry processing plants were able to control campylobacters on carcasses better than others. Neck skins collected post chill were one of the process stages evaluated by MO1055 and the results of that testing were shared with this study.

In addition, both the MO1055 study and this project undertook joint discussions with industry to agree a standardised test sample for an industry-led monitoring programme. The agreed sample comprised of three pooled neck skins. In total, there were 16 slaughterhouses across the UK that undertook testing of post chill neck skins and the results of these tests were collected as part of the current study. The quality of the laboratory testing was assessed by proficiency testing, also undertaken as part of this study. Testing laboratories with three or more calculation errors or outlying results per round were assessed as inadequate and their donations were excluded from the results dataset.

Finally, as part of separately-commissioned work, FSA employees collected post-chill neck skins samples as part of a baseline survey to determine the degree and prevalence of contamination of broiler carcasses in the UK. Testing of these samples was undertaken by the Animal Health Veterinary Laboratories Agency (AHVLA) in Weybridge (now renamed as Animal and Plant Health Agency [APHA]). The test results were also collected by this study; surveillance used in this project extended from April 2012 to June 2014.

Questionnaires matched to each test result. The questionnaires used for this project were created after in-depth discussions with UK processors. There were and initial two meetings with processing industry representatives and five meetings with technical staff in individual processing plants, where draft versions of the questionnaires were discussed and refined. It is particularly important to note that this study was heavily reliant on the cooperation of the UK processors and that a key issue for industry was the information used to answer the questionnaires should not require excessive amounts of staff effort to collate and supply. The approach of using multiple questionnaires for this study was a strategy agreed with industry to minimise asking for information that was unlikely to change on multiple occasions. The questionnaire contents were also influenced by knowledgeable industry representatives both from a technical viewpoint and as a strategy to prevent poor responses by using easy to obtain information. It is also important to note that the questionnaires were a negotiated balance between the goodwill of the UK poultry processing industry and the investigatory aspirations of the research team.

For each test result collected, food business operators (FBOs) were requested to donate a corresponding amount of supplementary information for use in the identification of risk factors. In brief, the additional information comprised:

- Farm infrastructure; which asked four questions relating to a farm **only once**. The answers to these questions were **unlikely to ever change** (e.g. What was the farm postcode? and County Parish Holding number?).

- Flock Information; which asked 14 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** for each batch.

- Plant infrastructure; which asked 44 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 ever change** (e.g. Was bird stunning by gas or electricity? How many plucker banks does the line have?).

- Plant Operations during the processing of individual slaughter batches; which asked 16 questions relating to processing conditions in the plant at the time the 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 asked for **each batch of birds** processed, because the answers could differ for each batch.

- Plant operations on sampling day; which asked six questions relating to processing conditions in the plant on the day of slaughter and was designed to minimise duplicate information where multiple batches of birds were sampled in a single processing day (e.g. How many days has it been since the chiller was last cleaned?). These questions were asked **once for each processing day**, even if multiple flocks were sampled on a single day.

The questionnaires used for the capture of the supplementary information collected for each plant, farm and processed batch of birds sampled are provided as **Appendix 6**.

Development of the on-line data collection and reporting facilities. The questionnaires (**Appendix 6**) 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 FSA and an industry-government steering committee called the Joint Working Group (JWG), and routine visits to 23 processing lines in 16 different poultry slaughterhouses were then made by the project team. Processor technical staff used a web browser to visit the website and 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 part of an established integrator company that was willing to provide information describing their farms and flocks for *en masse* risk factor identification. Information from related FSA-funded work (project reference FS101123) that involved smaller farms and processors was used as the study progressed. The

website was organised in a manner that automatically guided each approved user through the processing plant infrastructure questionnaire once only. Plants were provided with a choice of methods for the entry of neck skin laboratory test results. Periodic batches of test results were accepted by email *en masse* in a variety of spreadsheet formats. Plants could also manually type in test results. Both methods triggered email-based automated systems that prompted for farm descriptions, batch-specific flock growing conditions and also asked about the conditions during processing.

The technical details of the website were that it was built using the Microsoft (MS; Redmond, WA, USA) ASP.NET framework v2.5 on a webserver running the MS Server 2008 operating system. The site used cookie-less coding strategies such as the use of an encrypted page viewstate to hold data, in preparation for an EU e-privacy directive 2009/136 adopted in the UK on 25th May 2011. Customised active webpages were coded using either the C#.NET or VB.NET programming languages, and the questionnaire-response data was collected and stored in an instance of the MS-SQL (structured query language) database programme version 2008. All data saved and retrieved from the database was as parameterised, HTML-en/de-coded queries that prevented malicious script injection into the database and unintended manipulation of the page script. Technically-advanced coding methodologies such as dynamic page control placement during the page load event (**Figure 9**) and retrieval of dynamic control data from the page viewstate were used to make the site as easy to use as possible for processors.

Identification of risk factors for *Campylobacter* in chicken broilers project

Previously, you told us you had 8 sheds. Please can you tell us some basic information about these sheds?
To save you time, if the answers in any of the rows are all the same for each shed, just select the correct answer in the first column and then check the little box at the right of the question. The computer will then make all the selections in a row the same as the one chosen in first column when you click the 'save details' button.

Broiler shed details

Shed number		1	2	3	4	5	6	7	8
What was the approximate **age of the house/shed** that the sample was collected from?	All answers the same in this row	1 Years	1 Years	1 Years	1 Years	1 Years	1 Years	1 Years	1 Years
What **type of ventilation** was installed on the house/shed? i.e ridge extraction, tunnel, side wall, natural ventilation?	All answers the same in this row	Ridge extraction	Ridge extraction	Ridge extraction	Ridge extraction	Ridge extraction	Ridge extraction	Ridge extraction	Ridge extraction
How was the **house/shed constructed**?	All answers the same in this row	Metal frame	Metal frame	Metal frame	Metal frame	Metal frame	Metal frame	Metal frame	Metal frame
What **type of floor** did the house have?	All answers the same in this row	Concrete	Concrete	Concrete	Concrete	Concrete	Concrete	Concrete	Concrete
What litter was type is normally used in this shed?	All answers the same in this row	Sand	Sand	Sand	Sand	Sand	Sand	Sand	Sand

Save details   Main menu

Figure 9 Dynamic control placement was used to create customised data entry forms on a farm-by-farm basis to make the site easy-to-use for users. In the figure above, the form for the shed detail data was dynamically-created in response to a previous question that asked 'How many sheds are there on the farm?'.

Modelling to identify factors that predicted the numbers of campylobacters on broiler neck skins. In preparation for statistical analyses, the information in the relational database was required to be combined into a single flat-form spreadsheet. The general approach to assembling the information in the required format was to begin with the slaughter batches. For each slaughter batch, the corresponding farm information was linked to it in a single data table. A number of FBOs take birds from farms typically in a repeating cycle of between 45 and 55 days. Thus, there were some instances where different batches of birds were taken from different 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 county parish holding (CPH) number (or the red tractor assurance number as decided by the FBO), the FBO plant operations identifier (formerly the plant licence number). The structured query language (SQL) used to fetch the linked information is provided as **Appendix 7**.

Information collected by the website was held in the database tables as standard English language ASCII (American standard code for information interchange) 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 nominal or categorical. Nominal 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 categorical variables, there was no relationship between the value of the number and the information encoded. Numerical information such as bird age in days or house age in years was used without further conversion. Data were sorted in MS Excel (version 2010; Microsoft Corp, Redmond, WA, USA) 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 model, a two-level hierarchy was specified as the slaughterhouse and supply farm identifiers. In subsequent models, a broiler house identifier was included 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 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 $\alpha \leq 0.05$, using a Chi-square 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.

The model was developed starting from a base model which included a constant and a categorical variable which specified an additive effect for each sample collected. A key to describe the shortened variable names presented in the analysis is included towards the end of **Appendix 6**.

Microbiological testing to determine *Campylobacter* numbers on broiler neck skins. 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 304mm x 177mm stomacher bag (Seward, Thetford, England) inside out over a gloved hand, selecting carcases with neck skins of an appropriate length and excising a 10g sample using a pair of sterile scissors into the stomacher bag. Three combined neck skins were processed as a single sample.

Nine volumes of maximum recovery diluent (MRD, Oxoid, Basingstoke, UK) were added to each sample before homogenisation for 1 minute using a stomacher (Model number BA 6021, Seward, UK). *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^{o}C for 48h. Confirmation of *Campylobacter* spp. was by microscopic examination of 5 colonies per plate to confirm corkscrew motility; an inability to show visible growth at 25°C after 72h, positive testing for oxidase and the inability to ferment lactose and sucrose. Bacterial numbers on all decimally-diluted plates were converted into cfu g-1 or cfu ml-1 as appropriate according to the criteria described by ISO 6887-1:1999.

Modelling to identify factors that predicted the numbers of campylobacters in broiler house litter. In essence, the general approach taken to determine risk factors for flock colonisation was analogous to that used for the prediction of numbers of campylobacters on neck skins. The main difference was the sample type used for the farm studies and the test methodology. The sample tested was broiler house litter, collected by farm staff walking through the broiler house wearing Tyvek overshoes worn over disposable polythene overshoes. Detailed sample collection instructions have been previously published (FSA study FS241049 final project report; (Madden *et al*., 2014)). Litter-derived samples were tested by quantitative polymerase chain reaction (qPCR).

qPCR. Each reaction was supplied from the manufacturer as a lyophilised mix of primers and labelled probe, which was reconstituted as a master mix. 5µl of the master mix, was aliquoted into each test well of a 96 well RT (real time) PCR plate (Life Technologies Waltham, MA, USA). qPCR plates were sealed with adhesive film (MicroAmp Optical Adhesive Film, Life technologies) and briefly centrifuged (5000g, 30s) before thermal cycling. Reactions were undertaken on an Applied Biosystems ABI7500 instrument running 7500 fast systems sequence detection software (v1.4.0.27). The instrument cycling conditions were an initial heat to 95°C for 5 minutes to activate the HotStarTaq Plus DNA Polymerase. Followed by 40 cycles of a three-step amplification cycling:

- Denaturation, 15s at 95°C
- Annealing, 23s at 60°C, with data collection at 60°C
- Extension, 10s at 72°C

The detection reporter excitation and emission channels for *Campylobacter* DNA were 495 and 520 nm respectively. The internal controls used excitation at 524nm and detection at 557 nm.

As part of FSA study FS241049, a calibration curve was constructed for the qPCR assay by plotting the detection cycle values of the *Campylobacter* DNA standards against known numbers of cells. The range spanned six decimal dilutions of a *Campylobacter* type culture ($1x10^7$ to $1x10^2$ cfu/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.

RESULTS AND DISCUSSION

DATA MANIPULATIONS PRIOR TO ANALYSES

Critical inspection of the raw data revealed there were several issues that were required to be resolved prior to multivariate statistical analyses. The first issue related to inadvertent duplication of data entry. In many instances, FBOs had entered the same information more than once. Data entry errors of that type were readily identifiable if the information were ordered by sample collection date and grouped by the FBO plant operations identifier. 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).

The majority of duplicate entries were identical copies (n=18), however, there were five instances of duplication where the information was not identical. These instances were handled on a case-by-case basis. In one case, an early information save contained answers to all of the questions asked. However, a second save less than 8 seconds later had some of the answers changed to “Not answered”. The original save was used in that instance.

There were three instances of ‘accidental save’ where the questions at the beginning of the form had been answered, but those towards the end of the questionnaire had not been completed (identified by saving the default response for the later questions). In those instances, the latest version of the saved information was used.

One processor appeared to be providing deliberately unreliable information. An example of the type of errors that were evident in the submissions from the plant are shown as **Table 15**. The same CPH number was used for multiple farms over a period of several months. The FBO had been a reluctant participant and retailer/FSA intervention had been required to clear two large data backlogs, each of several months.

Since the provision of unreliable information had the potential to derail the whole modelling process, all of the information supplied by the FBO in question was excluded from the analyses as a unanimous decision of the project team (including the FSA project officer). After parsing to remove duplicate and suspicious data, the dataset was imported into the MLwiN software for statistical analyses.

Table 15 Selected example data from a single FBO that entered a single farm CPH number for farms with different postcodes (yellow highlight) and different CPH numbers for a farm with the same postcode (green highlight).

FarmCPH	WhenSaved	FarmPostcode	DistanceToPlant
290	06/11/2012 15:12:55	PE37 7QA	2783
290	06/11/2012 15:23:11	NR15 2YD	1313
1181	07/11/2012 02:12:22	GL2 8AA	6143
1476	07/11/2012 10:24:25	NR9 4NR	869
310	08/11/2012 09:43:32	NR28 0LU	3128
3163	08/11/2012 09:57:17	NR17 1DZ	711
290	08/11/2012 10:09:26	NR21 7HW	3152
307	08/11/2012 15:56:32	NR17 1AN	278
1746	08/11/2012 16:09:31	PE37 7QA	2783
290	08/11/2012 16:13:31	PE37 7QA	2783

RESTRICTIONS OF THE STATISTICAL PROCESS

The approach of the current study was to record information about already-operating farm and processing plants and to analyse that information to determine if there were factors that influenced the numbers of campylobacters. In recent years, the collection of data, and in some cases, big data, and statistical analyses has become an accepted scientific method for the identification of factors influencing a target of interest. The range of applications includes effective disease treatment identifications, better strategies for educating children and risk prediction for insurance industries. Although it is now firmly established, the approach is not perfect. In the current study, our role was to observe systems already operating to identify risk factors. Specifically, no attempt was made to ensure there were broadly equal numbers of different responses across the datasets. For the initial analyses, some of the responses provided were unevenly balanced e.g. there were only around 150/1780 sheds that contained beetles at harvest for the independent farm studies. In order for the statistics to be able to make credible comparisons, a larger number of sheds containing beetles would strictly be required.

In addition, for those factors where there was sufficient representation across all responses our identification of specific risk factors involved an original assessment to identify a broad area of risk, followed by more detailed investigations. Some attempt was made for this study to investigate the basis for the observed elevated and protective risk factors. However, these efforts focussed mainly on the published literature and since the study aims were to identify previously unknown risk factors, little further detail that was concrete was identified. Although the experience of the project team was that the participating farmers and processors were enthusiastic, the response rates to some questions were quite low. For example, for the independent farm studies, there were around 400 positive responses made that probiotic supplements had been used when growing birds, but less than 200 follow-on responses describing the type of prebiotic used.

A typical drawback of the statistical process is that it can identify factors that are correlated with *Campylobacter* numbers, but does not provide much explanation of the reasons for the association. As a general strategy, modelling is useful to

identify promising factors as a precursor to an experimental investigation that is balanced and appropriately replicated. Consequently, any discussion of the current study findings might be open to a criticism of being speculative. However, bearing that in mind, there was considerable discussions with farmers and knowledgeable industry representatives regarding the study findings. There is much that seems to be credible explanation of what may be occurring in plants, and on farms, and so should be recorded and discussed because it may be helpful to others working to tackle the same issues.

As an initial step, a Poisson model was constructed from the hierarchical supplementary information linked with the neck skin test result. In total around 1250 rows of information were initially collected. When those rows that did not have a *Campylobacter* count above the LoD of the test method (10 cfu/g) were removed, 964 rows of information remained. This data was used to undertake an initial run, in which only the seasonal variation was factored in to the model. It is already well established that counts follow a broadly sinusoidal pattern, linked with season. The results of which are shown as **Figure 10**.

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

$$\text{LogCount}_{ij} = \beta_{0ij}\text{Const} + -0.183073(0.047013)\text{Sin}_{ij} + 0.093399(0.044073)\text{Cos}_{ij} + -0.000329(0.000168)\text{TofYear\^{}1}_{ij}$$

$$\beta_{0ij} = 3.104814(0.161227) + u_{0j} + e_{0ij}$$

$$\left[u_{0j}\right] \sim \text{N}(0,\ \Omega_u)\ :\ \Omega_u = \left[0.209683(0.079081)\right]$$

$$\left[e_{0ij}\right] \sim \text{N}(0,\ \Omega_e)\ :\ \Omega_e = \left[0.676261(0.031085)\right]$$

*-2*loglikelihood(IGLS Deviance)* = 2404.186035(964 of 1077 cases in use)

Figure 10 The results of an initial run of a Poisson regression within a multilevel framework 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.

An important finding from this initial model was that it quantified the total variation described by the data and also the gross factors influencing the numbers of campylobacters on neck skins in processing plants. Overall, 0.886 total units of variance were identified by the model. 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 factors. Thus, although it was reported previously that slaughterhouses differ in their ability to hygienically process colonised birds into carcasses (Anonymous, 2010b), what occurred during the rearing of the birds on farm was more than three times more important in terms of the final carcass *Campylobacter* load. The errors between the actual plate count and the model prediction are shown in **Figure 11**. The numbers on the y-axis are the residual difference between the measured count and model predicted count. These were typically of the order of 0.1-0.2 log cfu/g neck skin on counts of up to five or six log cfu/g.

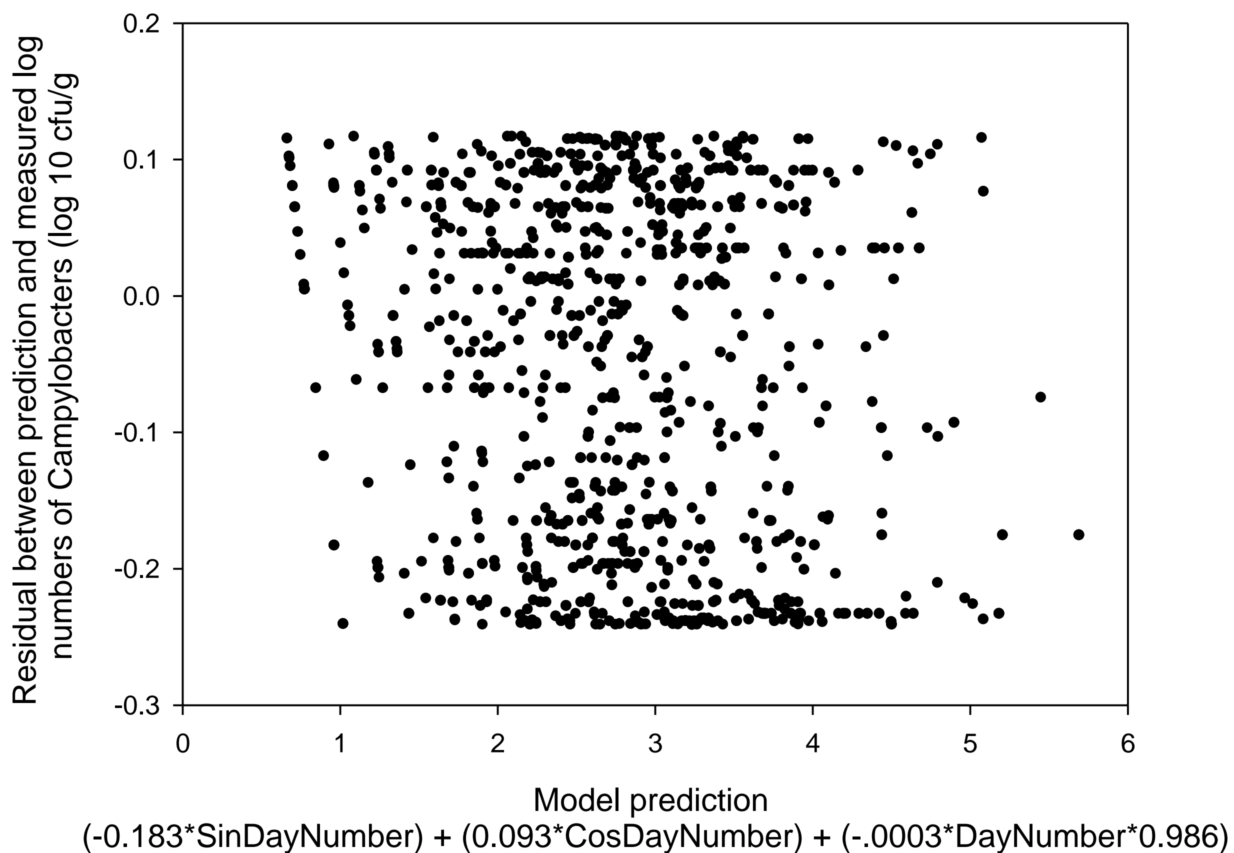


Figure 11 A visual depiction of the differences between the counts predicted by the model shown in **Figure 10** and the measured numbers of campylobacters on chicken neck skins.

The model was expanded by the addition and removal of factors to account for the observed variation. An emphasis was placed on slaughterhouse factors because there was a more comprehensive dataset associated with birds during processing compared with rearing. In addition, the identified importance of the on-farm factors meant a more in-depth investigation would be appropriate. We investigated why there were less complete datasets for the farm data compared with the processing information. In brief, the result of discussions revealed there was a widely-adopted organisational structure within processing plants. There were commonly two separate streams of personnel that have a single senior manager. The first stream was responsible for the processing of the birds within the plant. The second stream oversaw the agricultural aspects of chicken production such as feed and chick ordering and the scheduling of birds by weight or age to meet customer requirements. To answer the questions associated with farm infrastructure and batches of birds during rearing, the assistance of the agricultural team was required. However, the main interactions between the FSA, JWG and the industry were with the personnel stream that handled processing of birds. Whilst there was good ‘buy-in’ from the process stream staff, who reliably supplied the requested information relating to processing, the goodwill extended to asking but not pursuing agricultural staff for the growing phase information. The agricultural stream staff were not well informed

about the study or industry efforts to control campylobacters and so we believe they did not assign a high priority to the supply of farm data.

The final best-fit Poisson model is shown as **Figure 12**. A visual depiction of the differences between the model predictions and the measured numbers of campylobacters is shown as **Figure 13**. It is important to note that the straight line towards the left-hand side of **Figure 13** is a artefactual consequence of the limit of detection of 10 cfu/g of the ISO 10272-2 laboratory test method. The limit of detection imposes a non-zero baseline below which there were no counts.

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

$$\begin{aligned}\mathrm{LogCount}_{ij} = {} & \beta_{0ij}\mathrm{Const} + 0.3954526(0.2794164)\mathrm{IOCorrectiveActionsCompletionTime}_{ij} + 0.2345036(0.1140700)\mathrm{PostChillCTMZ}_{ij} + \\ & 0.1334945(0.0437489)\mathrm{PluckEffectivenessCriteriaMet}_{ij} + -0.2308706(0.1030306)\mathrm{ChillerCleanFreqMet}_{ij} + -0.1890836(0.0472284)\mathrm{Sin}_{ij} + \\ & 0.1163740(0.0443917)\mathrm{Cos}_{ij} + -0.0003337(0.0001676)\mathrm{TofYear}_{ij}\end{aligned}$$

$$\beta_{0ij} = 2.9792702(0.2853364) + u_{0j} + e_{0ij}$$

$$\left[u_{0j}\right] \sim \mathrm{N}(0,\ \Omega_u)\ :\ \Omega_u = \left[0.1572533(0.0607311)\right]$$

$$\left[e_{0ij}\right] \sim \mathrm{N}(0,\ \Omega_e)\ :\ \Omega_e = \left[0.6634510(0.0306290)\right]$$

*-2*loglikelihood(IGLS Deviance)* = 2361.8461608(956 of 1077 cases in use)

Figure 12 A Poisson regression undertaken within a multi-level framework describing the sources of variance influencing carcass *Campylobacter* load in UK slaughterhouses. Parameter estimates are in green with their standard errors shown within the brackets. 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 slaughterhouses and farms respectively and depict the respective impact of each term to the observed variance. Sin and Cos are transformations of TofYear (the sequential day number) mapped to 360 degrees.

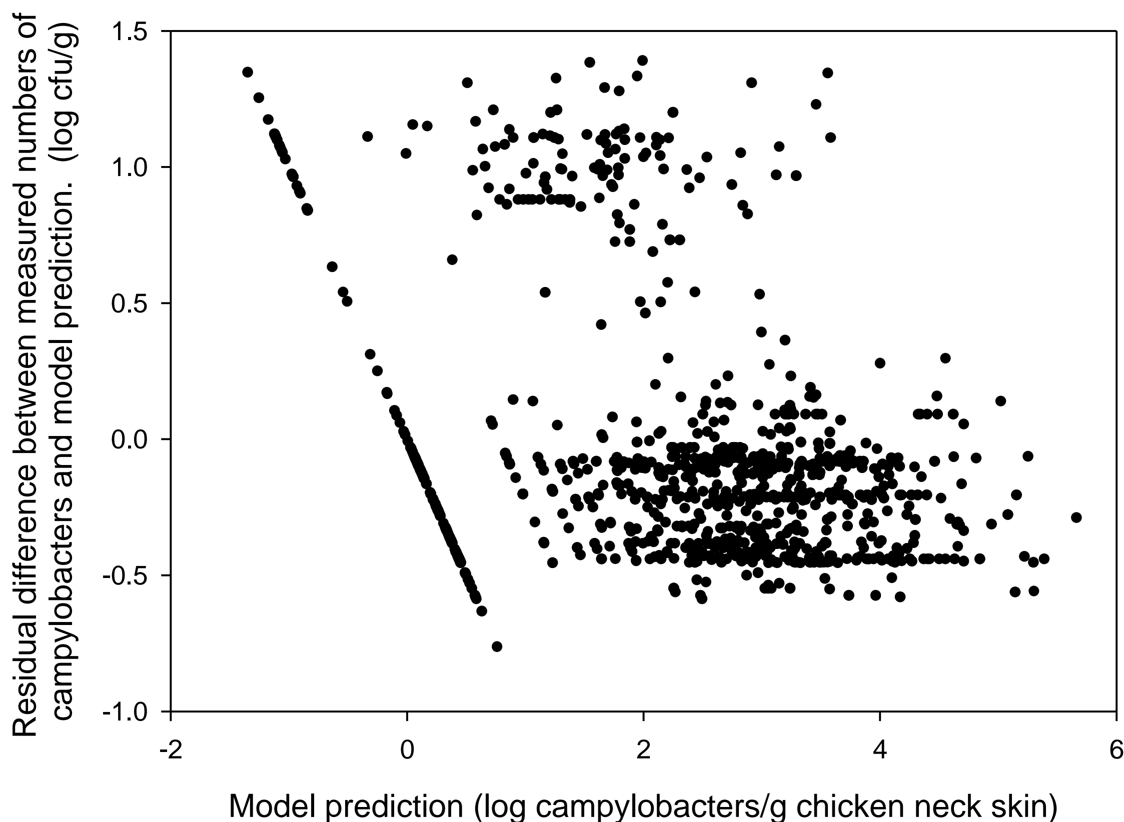


Figure 13 A chart showing the residual difference between the slaughterhouse operations model shown in **Figure 12** and the measured numbers of campylobacters on chicken neck skins.

The processing model revealed several variables that had significant influence on the numbers of campylobacters contaminating post chill neck skins. It has been previously reported that effective cold water washing causes typical reductions in *Campylobacter* numbers from 2.58 log cfu/ml chicken carcass rinse to 1.15 log cfu/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. In the final version of the model however, the time taken to implement corrective actions to ineffective IO washing fell just outside the threshold for significance. However, there were other factors that were significant which included the post chill carcass temperature (P=0.032); with lower temperatures associated with fewer campylobacters. It is also established that freezing chickens (Haughton *et al*., 2012) and chicken meat (Harrison *et al*., 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 can cause freeze-mediated stress and cellular death. The likely mechanism 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 pockets caused dissolved molecules to be displaced into the surrounding unfrozen fluid, thereby increasing the osmotic potential of that liquid (Dumont *et al*., 2004). As the extracellular fluid became more concentrated, it began to remove water from the cytoplasm of the campylobacters by osmosis (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 crystal formation (Dumont et al. 2004). In addition, there is evidence that superoxide radicals form during freezing, which are similarly concentrated in unfrozen pockets of water 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 presence of faecal leakage after plucking and the numbers and degree of carcass damage. Meeting the plucking effectiveness target was identified by the model as beneficial in terms of reduced *Campylobacter* load on carcasses (P=0.0082). 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;Allen *et al*., 2003b;Allen *et al*., 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 and colleagues (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 because of the plucking stage. Given the Takahashi conclusion, it seems reasonable to assume the model has identified that properly-adjusted plucking equipment might minimise cross-contamination of campylobacters between different flocks (Takahashi *et al*., 2006).

The model also identified the chiller cleaning frequency being met as important (P=0.018). Chiller cleaning is notoriously difficult to accomplish in poultry processing plants because the chillers are seldom empty, and processing typically occurs 18-20 hours per day. Air chilling is almost exclusively used in broiler processing in the UK and there are reports that chilling can reduce the numbers of *Campylobacter* measured from carcasses. Reductions in the numbers of campylobacters was observed in six out of 10 batches of birds examined just before and just after chilling (Allen *et al*., 2008). However, although the observed reductions were significant (P<0.001), 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 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 the storage to allow for survival of *Campylobacter* and to withstand the drying process that occurs at the same time as chilling. Although the model has identified chiller cleaning frequency as a risk factor, there is currently little in the literature to explain the mechanisms operating.

There are many reports of the seasonality of *Campylobacter* colonisation 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 model 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 model was that there was a significant linear trend of reducing numbers of campylobacters across the entire study (P=0.045). The observed reduction was 0.0033 log cfu/g of neck skin per day.

A POISSON MODEL IDENTIFYING RISK FACTORS FOR THE COLONISATION OF BROILER CHICKENS ON INDEPENDENT FARMS

Since the farm phase was identified by the initial model as important, there was further activity to identify on-farm risk factors.

A significant difference between the initial work and the farm-focussed work was that a litter derived sample was used to assess the bird status and degree of any colonisation rather than a post-chill neck skin. Two different categories of farm were investigated. These were smaller, independent farms supplying lower-throughput integrated or independent processors. Typical processor line speeds were 4000-5,000 birds per hour. Integrated farms owned by high-throughput (10,000-12000 birds per hour) processing plants, were also examined.

The final independent farm model developed using MLWinN is shown as **Figure 14**.

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

$$\begin{aligned}\text{LogLoading}_{ijk} = {} & \beta_{0ijk}\text{Constant} + 0.331(0.013)\text{BirdAge}_{ijk} + -1.107(0.277)\text{BirdGender_2}_{ijk} + \\ & -0.785(0.339)\text{BirdGender_3}_{ijk} + 1.400(0.360)\text{chkPrebiotic_1}_{ijk} + -1.091(0.422)\text{NewFmCat_2}_{k} + \\ & -0.477(0.986)\text{NewFmCat_5}_{k} + 2.459(0.448)\text{NewFmCat_6}_{k} + -0.263(1.036)\text{NewFmCat_7}_{k} + \\ & 0.807(1.646)\text{NewFmCat_10}_{k} + -2.249(1.519)\text{NewFmCat_11}_{k} + 1.789(0.962)\text{NewFmCat_13}_{k} + \\ & -1.237(0.433)\text{NewFmCat_14}_{k} + 0.462(0.175)\text{HouseConstruction_2}_{jk} + \\ & 1.438(0.843)\text{HouseConstruction_3}_{jk}\end{aligned}$$

$$\beta_{0ijk} = -8.561(0.521) + v_{0k} + u_{0jk} + e_{0ijk}$$

*-2*loglikelihood(IGLS Deviance)* = 7616.578(1780 of 1780 cases in use)

Figure 14 The final model for independent farms produced from the MLwiN multilevel analysis. Parameter estimates are in green with their standard errors shown within the brackets. 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 farms, broiler houses and farm batches respectively and depict the respective impact of each term to the observed variance. Farm categories (NewFmCat_x) are listed in **Appendix 8**.

The small farm model calculated the terms exerting significant influence on the $\log_{10}$ *Campylobacter* count. 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 in **Figure 15** would be used:

$$\text{Log10 Count} = -8.561 + (0.331 \times 42 \text{ (days)}) - 0.785 + 0.462$$

Figure 15 An example of the use of the equation shown in **Figure 14**. Here the calculation shows the predicted (mean) log10 *Campylobacter* count for a flock of age 40 days for mixed gender birds, in a metal framed broiler house.

Overall the analysis in **Figure 14** showed that, between an age of 26 to 50 days, for every one-day increase in the age of a flock there was a mean increase in $\log_{10}$ *Campylobacter* litter counts of 0.331 cfu/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/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/g compared with sheds containing only male birds ($p = 0.020$). From a practical viewpoint on farm, both male and females will be placed into houses on the same day and will generally be sourced from the same breeding stock (i.e. the farmer will get males and females from the same parent flock). In around 50% of cases, the day-old chicks are delivered to independent farms with the males and females mixed together.

Whether the gender and age predictors were correlated was also 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 UK 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 because cocks achieve a better feed conversion ratio (FCR, an index of how effectively the energy content of feed is converted to bird muscle) compared with pullets (a young female chicken). 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 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.

In contrast to integrated processors, independent slaughterhouses tend to favour heavier weight birds because a higher percentage of carcasses tended to be boned out for breast fillet rather than sold whole. One further contributing factor might be that final clearance male birds may experience catching in their house as many as five times during their life. Initial thinnings will most likely involve the females before commencing on lighter males. The employees undertaking

catching are a risk factor for *Campylobacter* colonisation 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 colonised with *Campylobacter*.

For each day a bird was farmed, there was a mean increase in $\log_{10}$ *Campylobacter* numbers of 0.331 cfu/g litter and investigations were undertaken to make sense of that finding. As a result of discussions between members of the project team and integrated and independent processors, it became clear that most integrators will thin only once i.e. one thin, and then houses are fully cleared. Independent processors however, will practice multiple thinnings before a shed is cleared. Farms with very large sheds, such those containing more than 50,000 birds; might thin as many as 6-8 times before final clearance. The long clearance times are a consequence of independent processors servicing customers that want a range of weights between 1.35 kg live weight and 3.5 kg live weight and most independent processors having a relatively low processing capacity compared with integrated slaughterhouses.

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 ($p < 0.001$). Although there was a mean increase of 1.438 $\log_{10}$ numbers in type 3 (other frame type) construction houses compared with wooden framed houses, this was not statistically, significantly different ($p = 0.088$). An attempt was made to reclassify the originally-assigned farm categories to take account of farms with larger houses and determine any influence on $\log_{10}$ *Campylobacter* numbers. The attempt was partly successful, although it was not possible to identify house areas (only the bird numbers typically placed) for all farms from the information collected. Thus, testing for an interaction between shed size and age to determine whether the rate was different between different shed sizes was not possible. Independent farms supplying integrated processors would be expected to thin fewer times and these types of farm did have lower counts in their litter compared with farms supplying independent processors. We also observed that two categories of farms supplying two separate independent processors had counts which were significantly lower than the general group of independent farms supplying independent processors. The houses on these two farm groups were predominantly wood-framed and it was these two farm groups that were mainly responsible for a study finding of a protective effect for wood framing as the house material.

We hypothesise 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 larger numbers of birds, and so the protective effect of wood framing may simply be a proxy of numbers 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).

If prebiotics were fed to birds, the mean log_{10} count was increased by 1.400 ($p < 0.001$). Prebiotics are compounds that function in a wide-ranging manner. Generally, their mechanism of action is to influence gut microbiota either by providing a nutrient preferentially to a specific group of bacteria or specific inhibition of the growth of some bacterial groups. However, prebiotics can also influence immunological targets and adhesion to gut columnar epithelia and thereby indirectly promote or inhibit bacterial populations (Pourabedin and Zhao 2015). Further investigation of the nature of the risk for prebiotic use was not possible. Although a number of farmers responded that prebiotics had been used, there were too few specific products listed for robust analyses. The use of prebiotics and the mechanisms causing the increased risk are subjects that will be investigated as further work.

In the early stages of the study we observed significance for short feed withdrawal time and lower *Campylobacter* numbers. The significance did not extend to the end of the project or survive multivariate analyses. However, we noted during the study duration that every time birds were thinned, feed was withdrawn from all the birds in the house, not just those being thinned. Feed withdrawal stresses birds and so larger sheds with multiple birds will have birds that were stressed multiple times. As was stated previously modelling can provide clues to important factors and follow up work can be properly designed to investigate such clues. The early significance of feed withdrawal time may be a clue it is important and that the influence was masked because the sample representing higher risk, longer feed times was under-represented as a coincidental consequence of the types of farms that participated in the study.

Farm category also exerted an influence on log_{10} *Campylobacter* numbers. More specifically, 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/g ($p = 0.001$). Furthermore, there were two categories of farms supplying two different independent processors that had counts which were 2.459 log_{10} cfu/g lower ($p = 0.001$) and 1.237 log_{10} cfu/g lower ($p < 0.001$) 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 ($p = 0.062$).

A POISSON MODEL TO DETERMINE RISK FACTORS FOR BIRD COLONISATION ON INTEGRATED FARMS

For the integrated model, litter testing and supplementary data collections were undertaken from at least three different batches of birds 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 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.

A small number of edits were made to the data before it was used. All the sheds that were sampled had barriers in their anterooms and so these responses were excluded from the statistical analyses. In addition, the number of days that sheds were empty between consecutive batches of birds was unanswered on around 40% of the questionnaires and thus this data was also excluded from the analyses. It has been previously reported that extended periods between consecutive flocks is a risk factor for bird colonisation (Hog *et al*., 2016) as a likely consequence of poorly maintained biosecurity in unused houses. The final model, manually assembled by assessing the impact of the on-farm factors and developed using MLwiN is shown as **Figure 16**.

$$\text{Log10Campy}_{ij} \sim \text{N}(XB, \Omega)$$

$$\text{Log10Campy}_{ij} = \beta_{0ij}\text{Constant} + 0.087(0.015)\text{BirdAge}_{ij} + \text{-}0.826(0.303)\text{No}_{ij} + \text{-}0.761(0.582)\text{Yes}_{ij} + 0.808(0.190)\text{BeetlePresence}_{ij} + 0.032(0.008)\text{HouseAge}_{ij} + \text{-}0.510(0.163)\text{BootsDipped}_{ij} + 0.319(0.082)\text{SampleType}_{ij}$$

$$\beta_{0ij} = 0.314(0.673) + u_{0j} + e_{0ij}$$

$$\left[u_{0j}\right] \sim \text{N}(0,\ \Omega_u) \ : \ \Omega_u = \left[1.879(0.450)\right]$$

$$\left[e_{0ij}\right] \sim \text{N}(0,\ \Omega_e) \ : \ \Omega_e = \left[3.818(0.188)\right]$$

*-2*loglikelihood(IGLS Deviance)* = 3759.800(876 of 876 cases in use)

Figure 16 The final model produced for integrated farms by the MLwiN multilevel analysis. Parameter estimates are in green with their standard errors shown within the brackets. 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 broiler houses respectively and depict the respective impact of each term to the observed variance. The model calculated the terms exerting significant influence on the $\log_{10}$ *Campylobacter* count. The constant (β_0) in the equation is tied to the first category of each categorical variable and to a bird age of zero.

Overall, the analysis in **Figure 16** showed that between the ages of 26 to 50 days, for every one-day increase in the age of a flock there was a mean increase in $\log_{10}$ *Campylobacter* litter counts of 0.087 cfu/g litter ($p < 0.001$).

It has been reported previously, bird age was found to be a significant predictor of bird colonisation status. Within the Sahin *et al*., (2015) review there are at least six previous reports of correlations between bird age and *Campylobacter* colonisation. A number of explanations have been proposed including the breaking of shed biosecurity by the chicken catchers, seasonal and climate changes, the use of ventilators 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, historically at least; the breaking of biosecurity was credibly implicated with *Campylobacter* colonisation and bird age.

Also related to biosecurity breaches, farmers that excluded their dogs from entry into the broiler houses showed a protective effect of significantly decreased numbers of campylobacters in the litter ($p<0.05$). In the final iteration of the 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 ($p=0.06$). More generally, the presence of a dog on farm did not quite reach significance ($P=0.057$). The importance of dogs on bird colonisation status has previously been identified as important on 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) also report that animals such as cattle, dogs, wildlife and rodents were significantly associated with positive flocks, whilst other studies have reported that farms with non-poultry livestock in close proximity (<2km, (Jonsson et al. 2012)) and the density of non-poultry farming operations near to broiler farms (Sommer *et al*., 2013) are risk factors. Although there are reports of other livestock increasing broiler colonisation 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 protective for campylobacters in house litter. Campylobacters can be ubiquitous environmental inhabitants and, in general, bacteria, including campylobacters, thrive 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 contains 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 colonisation. 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 significant until dogs in sheds were included into the model, the presence of darkling beetles (*Alphitobius diaperinus*; also, commonly referred to as the lesser mealworm) in the litter was a significant risk factor ($p<0.01$). Japp *et al*., (2010) report that a high degree of litter wetness is a risk factor for the colonisation 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 survive well on the beetles and could not be isolated from them one week after inoculation (Hazeleger *et al*., 2008). The Hazeleger study also collected limited numbers of naturally-contaminated insects from a commercial broiler farm and showed that when birds consumed these insects they also became colonised,

albeit at low levels. A general conclusion of the work was that *Campylobacter* can be transmitted via beetles and their larvae, and the authors emphasised it was a likely scenario that such transfers applied to successive rearing cycles.

House age was also identified as a risk factor, with older houses being more likely to contain colonised birds. Recently, Hog *et al*. (2016) identified the same risk factor, although their binomial model compared housed older and newer than five 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. Whilst that seems plausible, our observations are that older houses can have a less hygienic fundamental design and can be difficult to effectively clean and sanitise. 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 houses have a layer of dust on these supports that is several years old. There are broiler houses still in operation in the UK that pre-date 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 the protective effect of modern broiler houses.

The final risk factor identified by the model was sample type. Sample type is probably a proxy for bird age because the options of first clearance, second or later clearance and final clearance tend to occur at specific bird ages. The model reported that there was increasing colonisation risk with each subsequent clearance. Several 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.

GENERAL CONCLUSIONS FROM THE MODELS

As discussed above, industry has provided some credible explanations as to why some of the on-farm factors identified influence *Campylobacter* numbers in litter. However, to confirm/investigate the mechanisms that are operating, further experimental work should be commenced. Some of the required studies could be undertaken at small expense, by making use of existing standard operations within some companies. For example, there is at least one integrated processor in the UK that routinely sexes and grows birds in single sex houses on the same farm. The establishment of sample collections and testing on a series of suitable farms could form the foundation of further investigations to confirm our hypotheses or otherwise determine the nature of the protective effect for the female gender. Similarly, there were a small number of farms contained within the current studies that had a mixture of metal and wooden framed houses. Extended study of these farms and an appropriate balanced mixture of farms with exclusive different house frames might provide further clues regarding the nature of the protective effect of wooden framed houses on independent farms.

In addition to the factors that remained significant through the multi-level, multi-variate modelling process, there were some factors that were significant for most of the duration of both farm studies but did not survive the final analyses. The most interesting of these was the length of time that feed was withdrawn from birds during thinning, with shorter

withdrawal times having a protective effect. As was previously stated, the current study was observational and no attempt was made to recruit a balance of farms with broadly equal numbers for each of the different answer options on the questionnaires. Consequently, feed withdrawal time may be an important consideration that was masked by answer bias in the participating farms. Given feed withdrawal is likely to stress birds, and feed is withdrawn from all of the birds in a shed (not just the ones being thinned), there may be merit in further investigation using a balanced range of withdrawal times. Possibly, the role of feed withdrawal could be investigated in isolation from the other stresses associated with thinning i.e. investigation of feed withdrawal without exposure to potentially-contaminated catchers or the stress of the catching process.

The final risk factor that could be investigated further is the use of prebiotics, which increased litter counts on average by 1.4 log cfu/g on the independent farms. No significant influence was noted for the integrated farm studies. Prebiotics are compounds that manipulate gut microbiota using a diverse range of mechanisms. A general poor response to questions asking the full details of probiotic products used hampered better investigation of the mechanisms promoting bird colonisation by campylobacters for the independent study. However, it should be straightforward to design experimental work that compares colonisation in birds grown with and without popular prebiotic products.

APPENDIX 6 THE QUESTIONNAIRES MATCHED WITH TEST RESULTS USED FOR RISK FACTOR IDENTIFICATION

THE FARM INFRASTRUCTURE QUESTIONNAIRE

Question	Data entry type	Allowed responses (where appropriate)	Response stored in variable
What was the farm CPH (county parish holding) number (please exclude the UK prefix and leave out any slashes e.g. 9016008509)?	Text box	4-10 digits. Only numbers permitted	FarmCPH
How long before catching is feed withdrawn from the birds?	Dropdown list	Not answered, Up to 4 hours, Between 4 and 10 hours, More than 10 hours	FeedWithdrawalTime
Does the farm have a code of best practice for farm biosecurity?	Dropdown list	Not answered, Not known, No, Yes	BestPracticeBiosecurity
Does the farm have a code of best practice for the catching process?	Dropdown list	Not answered, Not known, No, Yes	BestPracticeCatching
Does the farm have a code of best practice for catcher personal hygiene and training?	Dropdown list	Not answered, Not known, No, Yes	BestPracticeCatcherHygiene
Does the farm periodically cut the grass or the weeds between broiler houses?	Dropdown list	Not answered, Yes, No, No plants between the houses	BetweenHousing

Does the farm source its feed from an accredited feed mill?	Dropdown list	Not answered, Yes, No, Not known	FeedMillAccreditation
How far is it from the farm to the processing plant? (distance roughly in miles)	Dropdown list	Not answered, 10 miles or less, 11-20 miles, 21-30 miles, 31-40 miles, 41-50 miles, more than 50 miles	DistanceToPlant
What is the farm postcode (we need it so we can find out the air temperature for harvest days)?	Text box	Regular expression validation to match the Royal Mail rules for a valid postcode	FarmPostcode
Is the farm fully compliant to the Red Tractor Farm Assurance Poultry Scheme (formerly the ACP scheme)?	Dropdown list	Not answered, No, Yes, Not known	RedTractorAssuranceCompliant

THE FLOCK QUESTIONNAIRE

Question	Data entry type	Allowed responses (where appropriate)	Response stored in variable
How long were the house(s) empty before the birds were placed? (please answer in days)	Dropdown list	Not answered, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, More than 20 days, Different houses empty different times	HouseEmptyTime
What was the date that the birds were placed?	Text box	Format as dd/mm/yyyy. Please enter a date.	WhenPlaced
What was the date that the birds were harvested?	Text box	Format as dd/mm/yyyy. Please enter a date.	WhenHarvested

What was the date that the birds were processed?	Text box	Format as dd/mm/yyyy. Please enter a date.	WhenProcessed
What was the farm CPH (county parish holding) number (please exclude the UK prefix and leave out any slashes e.g. 9016008509)?	Text box	4-10 digits. Only numbers permitted	FarmCPH
Was the slaughter batch tested to determine its *Salmonella* infection status?	Dropdown list	Not answered, No, Yes, Not known	SalmonellaStatusKnown
If the slaughter batch was tested, what was the test date?	Text box	Format as dd/mm/yyyy. Please enter a date.	WhenTestedSalmonella
If the slaughter batch was tested and the result was positive, was it scheduled for processing at the end of processing or after a group of similar-sized birds?	Dropdown list	Not answered, Yes - end of processing, Yes - after similar-sized birds, Not tested, Not a positive flock, Not known if tested	ScheduledProcessing
Had the slaughter batch previously been thinned or was it a 'brood and moved' slaughter batch?	Dropdown list	Not answered, Yes - previously thinned, Yes - brood and moved, Yes - previously thinned and brood and moved, No, Not known	ThinnedOrBroodAndMoved
If the flock had been previously thinned, please enter the previous thin dates:			
Date of first thinning	Text box	Format as dd/mm/yyyy. Please enter a date.	WhenThinned1
Date of second thinning	Text box	Format as dd/mm/yyyy. Please enter a date.	WhenThinned2
Date of third thinning	Text box	Format as dd/mm/yyyy. Please enter a date.	WhenThinned3

Date of fourth thinning	Text box	Format as dd/mm/yyyy. Please enter a date.	WhenThinned4
Were the birds from a single house or were birds multiple houses sent at the same time?	Dropdown list	Not answered, Single house, Multiple houses	BirdSourceHouses
How many birds were in the house? OR If more than one house was cleared what was the average number of birds per house?	Text box	Only numbers permitted	BirdsInHouse
What was the gender of the birds?	Dropdown list	Not answered, Mixed, Female, Male	Bird|Gender
What farming method was used to grow the birds?	Dropdown list	Not answered, Standard, Free Range, Organic, Freedom, Other	FlockType
What was the average bird weight for the slaughter batch (in grammes please)?	Text box	Only numbers permitted	MeanBirdMass
On average, how many birds were there in each crate?	Dropdown list	Not answered 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25	BirdsInCrate
What best describes the weather on the farm at the time the birds were caught?	Dropdown list	Not answered, Raining heavily, Raining lightly, Misty or foggy, Dry but overcast and dull, Dry with cloudy sunshine, Dry and bright sunshine	HarvestWeather
Were there any non-conformities between sections AM9 and AM9.11 of the Red Tractor Scheme for this slaughter batch?	Dropdown list	Not answered, No, Yes, Not known	RedTractorNonConform

Question	Data entry type	Allowed responses (where appropriate)	Response stored in variable
Infrastructure (Transport and Lairage)			
Are the transport crates and modules cleaned (i.e. wet removal of gross detritus) according to a best practice code before they leave the slaughterhouse?	Dropdown list	Not answered, No, Yes- up to 40% of transporters, Yes- 41-60% of transporters, Yes- 61-80% of transporters, Yes- 81-100% of transporters	CratesWashed
Are the transport crates and modules cleaned then sanitised (i.e. treated with a chemical decontaminant) according to a best practice code before they leave the slaughterhouse?	Dropdown list	Not answered, No Yes- up to 40% of transporters, Yes- 41-60% of transporters, Yes- 61-80% of transporters, Yes- 81-100% of transporters	CratesSanitised
Has crate cleaning and sanitation been 'validated as effective'?	Dropdown list	Not answered, Not applicable, No, Yes	CratesValidated
Infrastructure (Live Hang)			
Are birds stunned using gas or electricity?	Dropdown list	Not answered, Electricity, Gas	StunMethod
If electricity, are there bird-calming measures such as breast comforters and/or subdued lighting used in the hanging area?	Dropdown list	Not answered, No Gas stunning is used, Yes- one measure, Yes- more than one measure	BirdCalmingMeasures

Is the hanging area physically isolated or screened from the rest of the plant?	Dropdown list	Not answered, No, Yes	HangingAreaScreenedFromPlant
Is the hanging area physically isolated or screened from the crate washing area or is there a dust extraction system or other method for preventing hanging area contamination by aerosols from the crate washer?	Dropdown list	Not answered, No, Yes,	HangingAreaScreenedFromCrateWashArea
Are the directions of air movements into and out of the hanging area known?	Dropdown list	Not answered, No, Yes	AirMovementsLairage
Infrastructure (The Line)	Dropdown list		
How many processing lines are there in the plant?	Dropdown list	Not answered, 1, 2, 3, 4, 5, More than 5	NumberOfLines
Are there automatic rehanging between all the sections of all the lines?	Dropdown list	Not answered, Yes, No	AutoRehang
Do the birds ever rub against each other when any of the lines turn or change height?	Dropdown list	Not answered, Yes, No	BirdsRubbing
What happens when carcasses fall off the line?	Dropdown list	Not answered, Washed then rehung, Put into the petfood bin	DroppedCarcassFate
Does condensation ever drip from a line onto the carcasses?	Dropdown list	Not answered, Yes, No, Sometimes	CondensationDripFromLine

Have the shackle and belt cleaning and sanitation procedures been 'validated as effective'?	Dropdown list	Not answered, Not applicable, No, Yes	HangingCleanValidated
Are staff movements restricted so they can enter the plant only at the clean (chiller) end of processing and can move only towards the dirty (lairage) end of processing?	Dropdown list	Not answered, Yes, No	StaffMovementRestricted
On average, how many staff move between the various processing areas (e.g. between the evisceration and stunning areas) of the plant in a typical day?	Dropdown list	Not answered, Less than two people moving per hour, About two people moving per hour, More than two people moving per hour	NumberOfStaffMoving
	Dropdown list		
Infrastructure (Autokill)			
Are the blades on the autokiller continuously washed?	Dropdown list	Not answered, Yes, No	AutokillBladeWash
Is there manual re-hang between kill and evisceration?	Dropdown list	Not answered, Yes, No	RehangKillEV
Infrastructure (Scalding)			
Are multi-stage scalding tanks used?	Dropdown list	Not answered, Yes, No	MultiStageScaldTanks
Is there a counter current of clean water used in	Dropdown list	Not answered, Yes, No	CounterCurrentInScaldTank

the tank(s)?			
Is a validated treatment such as a pulsed electrical field to reduce bacterial load applied to tank water?	Dropdown list	Not answered, Yes, No	ScaldTankWaterTreatment
Are the tanks fully emptied between production periods?	Dropdown list	Not answered, Yes, No	ScaldTankFullEmptyProdnPeriods
How frequently are tanks fully emptied?	Dropdown list	Not answered, More frequent than per production period ,Emptied daily, Emptied weekly, Never fully emptied	ScaldTankFullEmptyFreq
Are the tanks part emptied between production periods?	Dropdown list	Not answered, No, Yes	ScaldTankPartEmptyProdnPeriods
How frequently are tanks part emptied?	Dropdown list	Not answered, More frequent than per production period, Emptied daily, Emptied weekly, Never part emptied	ScaldTankPartEmptyFreq
Has the cleaning and sanitation of the scald tank been 'validated as effective'?	Dropdown list	Not answered, No, Yes, Not applicable	ScaldTankCleanValidated

Infrastructure (Plucking)

How many plucker columns are there?	Dropdown list	Not answered, More than three, Three, Less than three	PluckerBanks
Are plucker fingers checked for surface-perish in-between processing periods?	Dropdown list	Not answered, No, Yes- some of the time, Yes- all of the time	PluckFingerChecked
When surface-perished fingers are identified, are corrective actions (e.g. finger	Dropdown list	Not answered, No, Yes- some of the time, Yes- all of the	PluckFingerPerishCorrectiveActions

replacements) implemented?		time	
Are the directions of air movements into and out of the plucking area known?	Dropdown list	Not answered, No, Yes	AirMovementsPluckers
Has the cleaning and sanitation of the pluckers before the commencement of processing been 'validated as effective'?	Dropdown list	Not answered, Not applicable, No, Yes	PluckerFullCleanValidated
Has the cleaning and sanitation of the pluckers during processing (e.g. during breaks) been 'validated as effective'?	Dropdown list	Not answered, Not applicable, No, Yes	PluckerPartCleanValidated

Infrastructure (Evisceration)

Is evisceration manual or automatic?	Dropdown list	Not answered, Automated, Manual	EVType
Is there a rapid mechanism to feedback evisceration effectiveness back to the EV operator adjusting the evisceration equipment?	Dropdown list	Not answered, No Evisceration is manual, Yes	EVEffectivenessRapidFeedbackMethod

Infrastructure (Cropping)

Has the cleaning and sanitation of the cropper been 'validated as effective'?	Dropdown list	Not answered, Not applicable, No, Yes	CropperValidated

Infrastructure (Washing)

What are the number of spray washes for the process?	Dropdown list	Not answered, No washes 1-3, 4-5, 5-6, more than 6	NozzleNumber
What is the volume of water used to spray each bird? (millilitres of water per bird)	Text box	Only numbers permitted	WaterVolumeSprayedPerBird
Is the water used for carcass washing heated to at least 80ºC?	Dropdown list	Not answered, Yes, No	EightyDegreesWashWater
Is there a manual re-hang between final wash and chill?	Dropdown list	Not answered, Yes, No	RehangIOWashChill

Infrastructure (Post Mortem Inspection)

Is the cleaning of hands/gloves a 'validated as effective' procedure?		Not answered, Not applicable, No, Yes,	PMIHandGloveValidated

Infrastructure (Chilling)

Are water sprays used in the chiller?	Dropdown list	Not answered, Yes, No	ChillerWaterSprays
Does the chiller have a UV lightbox which illuminates carcasses with a UV dose 'validated as effective' in significantly reducing *Campylobacter* numbers or numbers of other enteric human pathogens per cm^{-2} or per g of carcass?	Dropdown list	Not answered, No, Yes	ChillerUVIlluminated

Are the chiller condenser coils continuously illuminated with UV light?	Dropdown list	Not answered, No, Yes	ChillerCoilsUVIlluminated
Are neck skins automatically trimmed as the carcasses enter or exit the chillers?	Dropdown list	Not answered, No, Yes - on entry, Yes - on exit	NeckSkinTrimOnChillerExit
Are the chiller cleaning and sanitation procedures 'validated as effective'?	Dropdown list	Not answered, Not applicable, No, Yes	ChillerSanitationValidated

Infrastructure (Worker Hygiene)

Who cleans and/or sanitises worker aprons at the end of the working day?	Dropdown list	Not answered, No formal cleaning done, The slaughter line workers, A specialist cleaner	WhoCleansAprons
What form of disinfection is used?	Dropdown list	Not answered, No disinfection done, Spray disinfection, Foam disinfection	ApronDisinfectionForm
What is the active agent in the disinfectant?	Dropdown list	Not answered, Chlorine or hypochlorite, Gluteraldehyde, Didecyl Dimethyl Ammonium Chloride, Lauryl Dimethyl Benzyl Ammonium Chloride, Another quaternary ammonium compound, Another disinfection agent	ApronDisinfectionActiveAgent

Infrastructure (General)

Is the slaughterhouse subject to unannounced third party audit (e.g. from retailers)?	Dropdown list	Not answered, No, Yes	UnannouncedThirdPartyAudit
Does the plant have a mechanism to feed back	Dropdown list	Not answered, No, Yes	GeneralFeedbackMechanismExists

down the line problems such as increased visible contamination that becomes apparent only from inspection at the end of processing?			
Is the plant partitioned off into discrete and separate sections, with the line running through hatchways in the partitions?	Dropdown list	Not answered, No, Yes	Compartmentalised
Do the longer neck skins drag across the bottom of these hatchways as the carcasses run through them?	Dropdown list	Not answered, No ,Yes, Plant not partitioned	LongNeckSkinsDrag
Where does the plant draw its water for general usage?	Dropdown list	Not answered, Mains water, Borehole water, Other water source	WaterSource
Does your plant (i.e. not a water company) treat the water used for bird washing?	Dropdown list	Not answered, No, Yes - chlorine dioxide, Yes - UV light, Yes - EO water, Yes - ozone Yes - reverse osmosis, Yes - more than one type of treatment	PlantPotableWaterTreatment
What is your plant's annual throughput (in numbers of birds processed)?	Dropdown list	Not answered, less than 100,000 100,001 to half a million, 500,001 to 1 million, 1,000,001 to 5 million, 5,000,001 to 10 million, 10,000,001 to 15 million, 15,000,001 to 20 million, 20,000,001 to 30 million, 30,000,001 to 50 million, 50,000,001 to 75 million, 75,000,001 to 100 million, More than 100 million	AnnualPlantThroughput
Does your plant harvest and sell chicken livers?	Dropdown list	Not answered, Yes - all the time, Yes - some of the time, No	LiversForHumanConsumption

Question	Data entry type	Allowed responses (where appropriate)	Response stored in variable
Flock Information			
What is the CPH (county parish holding) number of the farm where the flock was raised (please exclude the UK prefix and leave out any slashes e.g. 9016008509)? Hover here for more info	Text box	4-10 digits. Only numbers permitted	FarmCPH
What was the date that the processing of the flock commenced (dd/mm/yyyy)?	Text box	Format as dd/mm/yyyy. Please enter a date.	StartTimeProcessing
What time interval best describes the approximate time that the samples of neck skin for *Campylobacter* testing were collected?	Dropdown list	Not answered, Noon to 1:59pm, 2pm to 3:59pm, 4pm to 5:59pm, 6pm to 7:59pm, 8pm to 9:59pm, 10pm to 11:59pm, Midnight to 1:59am, 2am to 3:59am, 4am to 5:59am, 6am to 7:59am, 8am to 9:59am, 10am to 11:59am	TimeSampled
Process monitoring (Transport and Lairage)			
How long did it take to transport the birds from the farm to the lairage (i.e. time interval from the first bird into a crate on farm to the first crate stacked inside the lairage)?	Dropdown list	Not answered, Time is not known, Less than 1 hour, 1 hr or more but less than 2 hrs, 2 hrs or more but less than 3 hrs, 3 hrs or more but less than 4 hrs, 4 hrs or more but less than 5 hrs, 5 hrs or more but less than 6 hrs, 6 hrs or more	TimeBirdsInCrates

How long on average did the birds spent in their crates in the lairage before hanging (i.e. the time interval from unloading of the first crate from the transporter to shackling the first bird onto the line)?	Dropdown list	Not answered, Time is not known, Less than 1 hour, 1 hr or more but less than 2 hrs, 2 hrs or more but less than 3 hrs, 3 hrs or more but less than 4 hrs, 4 hrs or more	TimeInLairage

Process monitoring (The Line)

What was the line speed for the flock during processing? (birds per hour)	Text box	Only numbers permitted	LineSpeed
For the day of sampling, how many flocks were processed before the flock that was sampled? (NB: if the plant has two or more lines, only count the flocks previously processed on the line that the samples were taken from)	Dropdown list	Not answered, 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20	NumberOfFlocksPreviouslyProcessed
Was the line stopped for five seconds or more during the processing of the flock that was sampled?	Dropdown list	Not answered, Yes, No	ProcessingHalted

Process monitoring (Scalding)

What was the temperature of the water in the scald tank?	Dropdown list	Not answered, Less than 55oC ,550C or higher	ScaldTankWaterTemperature

Process monitoring (Plucking)

Were the pluckers cleaned (i.e. wet removal of gross	Dropdown list	Not answered, No, Yes -every break, Yes -some breaks	PluckersCleaned

detritus) during the processing period (e.g. during breaks)?			
Were the pluckers cleaned and sanitised (i.e. treated with a chemical decontaminant) during the processing period?	Dropdown list	Not answered, No, Yes -every break, Yes -some breaks	PluckersSanitised

Process monitoring (Evisceration)

Was the evisceration equipment adaptively adjusted to the average bird size on a near-continual basis?	Dropdown list	Not answered, No, Yes - up to 40% of the day, Yes - 41-60% of the day, Yes - 61- 80% of the day, Yes - 81- 100% of the day	EVAdjustment
Was evisceration effectiveness assessed (e.g. by bile staining/faecal contamination of carcasses or the build-up of viscera on evisceration equipment)?	Dropdown list	Not answered, No, Yes - up to 40% of the day, Yes - 41-60% of the day, Yes - 61- 80% of the day, Yes - 81- 100% of the day	EVEffectiveness
What was the plant air temperature at the evisceration stage during processing of the flock? (º Centigrade)	Text box	Only numbers permitted	AirTempInLairage

Process monitoring (PMI)

For post mortem inspection, how often were gloves changed or hands/gloves cleaned?	Dropdown list	Not answered, Less frequent than every 20s (or per bird), Every 20s (or per bird if this is longer)	PMIGloveFrequency

Process monitoring (Cropping)

Was the cropping equipment cleaned during processing (e.g. during breaks)?	Dropdown list	Not answered, No, Yes	CropperCleaned
Was the cropping equipment cleaned and sanitised during processing?	Dropdown list	Not answered, No, Yes	CropperCleanedSanitised

Process monitoring (Spray washing)

For the final wash:

Was the spray wash water treated using either chlorine or EO water?	Dropdown list	Not answered, Not known, No, Yes- every day, Yes- every shift, Yes- checked every hour	SprayWaterTreated

Post process monitoring (Chilling)

Were the chiller air temperatures recorded?	Dropdown list	Not answered, No, Yes for some flocks, Yes for all flocks	ChillerAirTempMeasured
What was the chiller air temperature?	Dropdown list	Not answered, Not recorded, 5oC or more, less than 5oC, Less than 0oC, less than -5oC, less than -10oC, less than -15oC, less than -20oC	ChillerAirTemp
Were carcass temperature profiles recorded?	Dropdown list	Not answered, No, Yes, for some flocks, Yes for all flocks	PostChillCarcTemp
Were the temperature profiles as expected to prevent the growth of enteric potential human pathogens such as campylobacters?	Dropdown list	Not answered, No, Did not record profiles, Yes	ChillerEffectiveness

How long were the carcasses from the sampled slaughter batch in the chiller?	Dropdown list	Not answered, Less than 1 hour, 1hr-1.5hrs, 1.6-2hrs, More than 2 hr, Not known	ChillTime
Were there carcasses touching other carcasses in the chiller (including during line direction changes)?	Dropdown list	Not answered, Yes, No	CarcassesTouching
Were there carcasses dripping water onto other carcasses stored underneath?	Dropdown list	Not answered, Yes, No	CarcassDrippage

Process monitoring (Post Chill)

How many size (or weight) classes of carcass were there in the flock?	Dropdown list	Not answered, Not known, 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, More than 10	BirdSizeNumber
What was the percentage of B grade birds in the batch? (percent)	Dropdown list	0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, More than 40, Not known	BGradeBirds

THE PLANT ENVIRONMENT CONDITIONS DURING THE PROCESSING DAY QUESTIONNAIRE

Question	Data entry type	Allowed responses (where appropriate)	Response stored in variable
Process monitoring (Live hang)			
Was there air movement from the hanging area into the plant?	Dropdown list	Not answered, Yes, No, Unknown	AirMovementLairageToPlant
Process monitoring (The Line)			
Did the line run faster than usual during the processing day?	Dropdown list	Not answered, Yes - up to 10% faster, Yes - between 11% and 20% faster, Yes - more than 20% faster, No	FastestLineSpeed
Process monitoring (Plucking)			
Were the pluckers cleaned (i.e. wet removal of gross detritus) before the commencement of the day's processing?	Dropdown list	Not answered, No, Yes	PluckersCleanedPreProcessing
Were the pluckers cleaned and sanitised (i.e. treated with a chemical decontaminant) before the commencement of the day's processing?	Dropdown list	Not answered, No, Yes	PluckersSanitisedPreProcessing

Was there air movement from the plucking area towards the clean end of the plant?	Dropdown list	Not answered, Not known, Yes, No	AirMovementPluckersToPlant
Were the plant's own processing limits for plucking effectiveness (e.g. plucker damage to viscera; broken bones etc.) met during the day's processing?	Dropdown list	Not answered, Yes, No, No plant processing limits at this stage	PluckerEffectivenessMet

Process monitoring (Evisceration)

Were the plant's own processing limits for evisceration effectiveness (e.g. bile staining, broken bones etc.) met during the day's processing?	Dropdown list	Not answered, Yes, No, No plant processing limits at this stage	EVEffectivenessMet

Process monitoring (Cropping)

Was the cropping equipment cleaned (i.e. the wet removal of gross detritus) before the commencement of processing?	Dropdown list	Not answered, No, Yes	PostSPFPCropperClean
Was the cropping equipment cleaned and sanitised (i.e. treated with a chemical decontaminant) before the commencement of processing?	Dropdown list	Not answered, No, Yes	PostSPFPCropperSanitise
Were the plant's own processing limits for cropping effectiveness (e.g. punching on target and not causing broken bones) met during the day's processing?	Dropdown list	Not answered, No, Yes	CroppingEffectivenessMet

Process monitoring (Spray washing)

For the final wash:

Were the nozzles checked to ensure they are not blocked and functioning effectively by providing an even spray of water every processing period?	Dropdown list	Not answered, No, Yes- every day, Yes- every shift, Yes- checked every hour	SprayNozzleCheck
If the wash system was not functioning in a satisfactory manner, were corrective actions immediately commenced?	Dropdown list	Not answered, No Wash system operation, not defective, Yes	WaterPressureCheck
How long did any corrective actions take to complete and restore the wash system to satisfactory operation?	Dropdown list	Not answered, Not yet completed, Completed within 1 hour, Completed before end of processing period, Wash system operation not defective	IneffectiveWashingCorrectiveActionsTime

Post process monitoring (Chilling)

How frequently were the chiller floors cleaned (i.e. the wet removal of gross detritus) in the week before the neck skins were taken for testing?	Dropdown list	Not answered, Less frequent than weekly ,On a weekly basis, On a twice weekly basis, On a daily basis	ChillerFloorCleaningFreq
How frequently were the chiller floors cleaned and sanitised (i.e. the application of a decontaminating chemical) in the week before the neck skins were taken for testing?	Dropdown list	Not answered, Less frequent than weekly, On a weekly basis, On a twice weekly basis, On a daily basis	ChillerFloorSanitiseFreq
How frequently were the chiller walls, ceiling and environment cleaned in the week before the neck skins were taken for testing?	Dropdown list	Not answered, Less frequent than weekly, On a weekly basis, On a twice weekly basis, On a daily basis	ChillerWallCeilingCleaningFreq
How frequently were the chiller walls, ceiling and environment cleaned and sanitised in the week before the	Dropdown list	Not answered, Less frequent than weekly, On a weekly basis, On a twice weekly basis, On a daily basis	ChillerWallCeilingSanitiseFreq

neck skins were taken for testing?			
Within the previous week, was there anything which happened that caused changes to either the frequency of chiller cleaning or the way in which the chillers were cleaned?	Dropdown list	Not answered, No, Yes	UnusualChillerCleanReason

Plant-related information (General)

To what processing date do these answers relate (dd/mm/yyyy)?	Text box	Format as dd/mm/yyyy. Please enter a date.	SamplingDate

APPENDIX 7 THE SQL USED TO CREATE THE TABLES USED TO STORE THE COLLECTED SUPPLEMENTARY INFORMATION AND HARVEST THE COLLECTED DATA

Farm, broiler house and bird batch information was stored in a relational database (MS SQL server 2008). Tables were created to hold data describing farms, broiler sheds, batch-specific flock information and laboratory testing results. The original tables were created by executing the following statements inside the SQL server programme. Please note that as the project advanced and the supplementary information questionnaires were developed, some additional column headings were added (and some were removed). The final version of the questionnaires and the column headings used to store the response information can be found in **Appendix 6**.

1. CREATE TABLE tblFarmDetails (UserName nvarchar(50), FarmName nvarchar(50), FarmAddress2 nvarchar(50), FarmAddress3 nvarchar(200), FarmAddress4 nvarchar(50), FarmAddress5 nvarchar(50), MobileNumber nvarchar(50), FarmCPH nvarchar(50), FeedWithdrawalTime nvarchar(50), NumberOfHouses nvarchar(50), GroundMaterial nvarchar(50), DoesThinning nvarchar(50), BestPracticeBiosecurity nvarchar(50), AccreditedFeedMill nvarchar(50), SalmonellaTestDone nvarchar(50), OtherSpeciesPresent nvarchar(50), WhenSaved datetime, ID INT NOT NULL IDENTITY PRIMARY KEY)

2. CREATE TABLE tblShedDetails (UserName nvarchar(50), HouseNumber nvarchar(50), VentilationType nvarchar(50), HouseConstruction nvarchar(50), HouseFloorType nvarchar(50), LitterType nvarchar(50), WhenSaved datetime, ID INT NOT NULL IDENTITY PRIMARY KEY)

3. CREATE TABLE tblFlockDetails (UserName nvarchar(50), SourceHatchery nvarchar(50), TimeHouseEmpty nvarchar(50), DatePlaced datetime, BirdAge nvarchar(50), SampleCollectionDate datetime, DayOldsPlaced nvarchar(50), LiveWeight nvarchar(50), ThinDate datetime, CDMR nvarchar(50), CDMRReasonHigh nvarchar(500), Breed nvarchar(50), PreviouslyThinned nvarchar(50), HouseNumber nvarchar(50), HouseAge nvarchar(50), BirdGender nvarchar(50), chkDrinkSan int, chkVitamin int, chkVaccination int, chkProbiotics int, chkPrebiotic int, chkOtherSupplement int, SpecificSupplements nvarchar(500), DiseaseDiagnosed nvarchar(50), SpecificDisease nvarchar(50), AntibioticsGiven nvarchar(50), SpecificAntibiotics nvarchar(500), BirdAgeAbGiven nvarchar(50), LitterWetness nvarchar(50), SalmonellaTestResult nvarchar(50), CrateDesign nvarchar(50), Slaughterhouse nvarchar(50), LicenceNumber nvarchar(50), AnythingElse nvarchar(500), WhenSaved datetime, ID INT NOT NULL IDENTITY PRIMARY KEY)

4. CREATE TABLE tblResults (FarmCPH nvarchar(50), DateSampled datetime, HouseNumber nvarchar(50), Results nvarchar(50), Loading decimal (12, 3), ReportingDate datetime, ID INT NOT NULL IDENTITY PRIMARY KEY)

5. CREATE TABLE tblPlantOpsData (SamplingDate datetime, AverageLineSpeed nvarchar(50), FastestLineSpeed nvarchar(50), TimeBirdsInCrates nvarchar(50), AirMovementLairageToPlant nvarchar(50), ScaldTankTemp nvarchar(50), PluckersCleanedPreProcessing nvarchar(50), PluckersSanitisedPreProcessing nvarchar(50), PluckersCleanedDuringProcessing nvarchar(50), PluckersSanitisedDuringProcessing nvarchar(50), AirMovementPluckersToPlant nvarchar(50), EVAdaptivelyAdjusted nvarchar(50), EVEffectivenessAssessed nvarchar(50), EVEffectiveness nvarchar(50), PMHandGloveFrequency nvarchar(50), CavityInspectionHandGloveFrequency nvarchar(50), WaterPressureCheck nvarchar(50), SprayNozzleCheck nvarchar(50), IneffectiveWashingCorrectiveActionsTime nvarchar(50), CorrectiveActionsCompletionTime nvarchar(50), SPFSprayTemperature nvarchar(50), USFSprayTemperature nvarchar(50), ChillerAirTempRecords nvarchar(50), CarcassChillTempRecords nvarchar(50), TemperatureProfileSatisfactory nvarchar(50), TouchingCarcasses nvarchar(50),

DrippingCarcasses nvarchar(50), ChillerFloorCleaningFreq nvarchar(50), ChillerFloorSanitiseFreq nvarchar(50), ChillerWallCeilingCleaningFreq nvarchar(50), ChillerWallCeilingSanitiseFreq nvarchar(50), SPFUnavoidablyProcessedPercentage nvarchar(50), PostSPFPluckerClean nvarchar(50), PostSPFPluckerSanitise nvarchar(50), PostSPFCropperClean nvarchar(50), PostSPFCropperSanitise nvarchar(50), PostSPFShackleBeltClean nvarchar(50), PostSPFShackleBeltSanitise nvarchar(50), LicenceNumber nvarchar(50), WhenSaved datetime, ID INT NOT NULL IDENTITY PRIMARY KEY)

6. CREATE TABLE tblPlantInfrastructureData (CratesCleanedBeforeLeaveSlaughterHouse nvarchar(50), CratesSanitisedBeforeLeaveSlaughterHouse nvarchar(50), ValidatedCrateWashSanitiseProcedure nvarchar(50), StunMethod nvarchar(50), BirdCalmingMeasures nvarchar(50), HangingAreaScreenedFromPlant nvarchar(50), HangingAreaScreenedFromCrateWashArea nvarchar(50), HangAreaAirMovesKnown nvarchar(50), AutoReHangBetweenLineSections nvarchar(50), BirdsRubbing nvarchar(50), DroppedCarcassFate nvarchar(50), CondensationDripFromLine nvarchar(50), ShackleBeltCleanSanitiseValidated nvarchar(50), AutoKillBladesWashedContinually nvarchar(50), ManualReHangBetweenKillAndEV nvarchar(50), MultiStageScaldTanksUsed nvarchar(50), CounterCurrentInScaldTank nvarchar(50), ScaldTankWaterTreatment nvarchar(50), ScaldTankFullyEmptiedBetweenProductionDays nvarchar(50), ScaldTankFullEmptyingFrequency nvarchar(50), ScaldTankPartlyEmptiedBetweenProductionDays nvarchar(50), ScaldTankPartEmptyingFrequency nvarchar(50), ValidatedScaldTankWashSanitiseProcedure nvarchar(50), PluckerFingerPerishCheckDuringProcessing nvarchar(50), PerishedFingerCorrectiveActions nvarchar(50), PluckAreaAirMovesKnown nvarchar(50), ValidatedPluckerCleanSanitiseBeforeProcessing nvarchar(50), ValidatedPluckerCleanSanitiseDuringProcessing nvarchar(50), EVAutoOrManual nvarchar(50), EVEffectivenessRapidFeedbackMethod nvarchar(50), ValidatedCropperWashSanitiseProcedure nvarchar(50), SprayWashNumber nvarchar(50), WaterVolumeSprayedPerBird nvarchar(50), EightyDegreesWashWater nvarchar(50), ManualReHangBetweenFinalWashAndChill nvarchar(50), ValidatedHandGloveCleanSanitiseProcedure nvarchar(50), ChillerWaterSprays nvarchar(50), ChillerCarcassUVLightbox nvarchar(50), ChillerCoilsUVIlluminated nvarchar(50), NeckSkinTrimOnChillerExit nvarchar(50), ValidatedChillerWashSanitiseProcedure nvarchar(50), UnannouncedThirdPartyAudit nvarchar(50), GeneralFeedbackMechanismExists nvarchar(50), PlantPartitioned nvarchar(50), LongNeckskinsDrag nvarchar(50), LicenceNumber nvarchar(50), WhenSaved datetime, ID INT NOT NULL IDENTITY PRIMARY KEY)

The tables were linked using the farm identifier, either the sample collection date (litter samples) or the date of slaughter (neck skin samples) and the broiler house number on a per user basis (i.e. on a slaughterhouse by slaughterhouse basis). In combination, these three keys were a unique sample identifier. Data were harvested for analyses by executing the SQL statement:

SELECT tblResults.FarmCPH, tblResults.DateSampled as ResultsDateSampled, tblFlockDetails.DateSampled as FlockDateSampled, tblResults.HouseNumber, tblResults.Results, tblFlockDetails.Username, tblFlockDetails.DatePlaced, tblFlockDetails.BirdAge, tblFlockDetails.SourceHatchery, tblFlockDetails.TimeHouseEmpty, tblFlockDetails.DayOldsPlaced, tblFlockDetails.CDMR, tblFlockDetails.CDMRReasonHigh, tblFlockDetails.Breed, tblFlockDetails.HouseNumber AS Expr2, tblFlockDetails.BirdGender, tblFlockDetails.chkDrinkSan, tblFlockDetails.chkVitamin, tblFlockDetails.chkVaccination, tblFlockDetails.chkProbiotics, tblFlockDetails.chkPrebiotic, tblFlockDetails.chkOtherSupplement, tblFlockDetails.SpecificSupplements, tblFlockDetails.DiseaseDiagnosed, tblFlockDetails.SpecificDisease, tblFlockDetails.AntibioticsGiven, tblFlockDetails.SpecificAntibiotics, tblFlockDetails.BirdAgeAbGiven, tblFlockDetails.LitterWetness, tblFlockDetails.AnythingElse, tblFlockDetails.Barrier, tblFlockDetails.WhenSaved, tblFlockDetails.BootsDipped, tblFlockDetails.DogInShed, tblFlockDetails.PreviouslyCaughtBirds, tblFlockDetails.SampleType, tblFlockDetails.BeetlePresence, tblFlockDetails.BirdsStressed, tblResults.Loading, tblResults.ReportingDate, tblFarmDetails.FarmName, tblFarmDetails.FarmAddress, tblFarmDetails.FarmTown, tblFarmDetails.FarmCounty, tblFarmDetails.FarmPostCode, tblFarmDetails.MobileNumber, tblFarmDetails.FarmCPH, tblFarmDetails.FeedWithdrawalTime, tblFarmDetails.DoesWaterDisinfect, tblFarmDetails.NumberOfHouses, tblFarmDetails.CycleLength, tblFarmDetails.GroundMaterial, tblFarmDetails.DoesFogging, tblFarmDetails.BestPracticeBiosecurity, tblFarmDetails.ProductionMethod, tblFarmDetails.Slaughterhouse, tblFarmDetails.LicenceNumber, tblFarmDetails.WhenSaved, tblFarmDetails.chkDog, tblFarmDetails.chkCat, tblFarmDetails.chkCattle, tblFarmDetails.chkSheep, tblFarmDetails.chkPig,

tblFarmDetails.chkTurkey, tblFarmDetails.chkHorse, tblFarmDetails.chkLlama, tblFarmDetails.chkOther, tblFarmDetails.Accepted, tblFarmDetails.FarmCPH, tblFarmDetails.FarmCategory, tblFarmDetails.AdditionalInfo, tblShedDetails.HouseAge, tblShedDetails.VentilationType, tblShedDetails.HouseConstruction, tblShedDetails.HouseFloorType, tblShedDetails.Littertype FROM tblResults full JOIN tblFlockDetails ON (tblFlockDetails.DateSampled = tblResults.DateSampled) AND (tblResults.HouseNumber = tblFlockDetails.HouseNumber) AND (tblResults.FarmCPH = tblFlockDetails.UserName) LEFT JOIN tblFarmDetails ON (tblResults.FarmCPH = tblFarmDetails.FarmCPH) LEFT JOIN tblShedDetails ON (tblResults.HouseNumber = tblShedDetails.HouseNumber) AND (tblFarmDetails.Username = tblShedDetails.UserName)

APPENDIX 8 FARM CATEGORIES USED FOR THE INDEPENDENT FARMS SUPPLYING LOWER-THROUGHPUT PROCESSORS

Farm category	Farm category description
1	Independent farms supplying independent processors in England
2	Independent farms supplying integrated processors in England and not currently litter testing for campylobacters
3	Independent farms supplying integrated processors in England and currently litter testing for campylobacters
4	Integrator-owned farms currently litter testing for campylobacters
5a	Integrator-owned farms supplying an integrated processor (Processor G) in a specific region of the UK and not currently litter testing for campylobacters
5b	Independent farms supplying an integrated processor (Processor G) in a specific region of the UK and not currently litter testing for campylobacters
6	Independent farms supplying an independent processor (Processor H) in a specific region of the UK and not currently litter testing for campylobacters
7	Independent farms supplying an integrated processor with multiple processing plants in a specific region of the UK and not currently litter testing for campylobacters
8	Independent farms supplying an independent processor (Processor J) in a specific region of the UK and not currently litter testing for campylobacters
9	Independent farms supplying an independent processor (Processor K) in a specific region of the UK and not currently litter testing for campylobacters
10	Independent farms supplying an integrated processor with multiple processing plants in a specific region of the UK and not currently litter testing for campylobacters
11	Independent farms supplying an integrated processor with multiple processing plants in a specific region of the UK and not currently litter testing for campylobacters
12	Independent farms supplying an integrated processor with multiple processing plants in a specific region of the UK and not currently litter testing for campylobacters
13	Independent farms supplying an independent processor (Processor L) in a specific region of the UK and not currently litter testing for campylobacters
14	Independent farms supplying an independent processor (Processor M) in a specific region of the UK and not currently litter testing for campylobacters

REFERENCES

Aburuwaida,A.S., Sawaya,W.N., Dashti,B.H., Murad,M. and Alothman,H.A. (1994) Microbiological Quality of Broilers During Processing in A Modern Commercial Slaughterhouse in Kuwait. *Journal of Food Protection* **57**, 887-892.

Adak,G.K., Meakins,S.M., Yip,H., Lopman,B.A. and O'Brien,S.J. (2005) Disease risks from foods, England and Wales, 1996-2000. *Emerging Infect. Dis.* **11**, 365-372.

Alali,W.Q. and Hofacre,C.L. (2016) Preharvest Food Safety in Broiler Chicken Production. *Microbiology Spectrum* **4**.

Allen,V.M., Hinton,M.H., Tinker,D.B., Gibson,C., Mead,G.C. and Wathes,C.M. (2003a) Microbial cross-contamination by airborne dispersion and contagion during defeathering of poultry. *British Poultry Science* **44**, 567-576.

Allen,V.M., Tinker,D.B., Hinton,M.H. and Wathes,C.M. (2003b) Dispersal of micro-organisms in commercial defeathering systems. *British Poultry Science* **44**, 53-59.

Allen,V.M., Weaver,H., Ridley,A.M., Harris,J.A., Sharma,M., Emery,J., Sparks,N., Lewis,M. and Edge,S. (2008) Sources and spread of thermophilic *Campylobacter* spp. during partial depopulation of broiler chicken flocks. *Journal of Food Protection* **71**, 264-270.

Anonymous. (2001). Strategic Plan 2001 – 2006, Putting consumers first. The Food Standards Agency, England. https://www.food.gov.uk/multimedia/pdfs/stratplan.pdf. Accessed 28.02.17

Anonymous (2003). UK-wide Survey of *Salmonella* and *Campylobacter* Contamination of Fresh and Frozen Chicken on Retail Sale. https://www.food.gov.uk/sites/default/files/b180002finreport.pdf. Accessed 28.02.17

Anonymous. (2007). Strategic Plan to 2010 - Putting consumers first. The Food Standards Agency, England. http://tna.europarchive.org/20110116113217/http://www.food.gov.uk/multimedia/pdfs/strategicplan2010e.pdf. Accessed 28.02.17

Anonymous (2010a). The joint government and industry target to reduce *Campylobacter* in UK produced chickens by 2015. https://www.food.gov.uk/sites/default/files/multimedia/pdfs/campytarget.pdf Accessed 28.02.17

Anonymous (2010b). Analysis of the baseline survey on the prevalence of *Campylobacter* in broiler batches and of *Campylobacter* and *Salmonella* on broiler carcasses in the EU, 2008. http://onlinelibrary.wiley.com/doi/10.2903/j.efsa.2010.1503/epdf. Accessed 28.02.17

Archer,D.L. (2004) Freezing: an underutilized food safety technology? *International Journal of Food Microbiology* **90**, 127-138.

Battersby,T., Whyte,P. and Bolton,D. (2016) Protecting broilers against *Campylobacter* infection by preventing direct contact between farm staff and broilers. *Food Control* **69**, 346-351.

Bauermeister,L., Bowers,J., Townsend,J. and Mckee,S. (2008) The Microbial and Quality Properties of Poultry Carcasses Treated with Peracetic Acid as an Antimicrobial Treatment. *Poultry Science* **87**, 2390-2398.

Berrang,M.E. and Bailey,J.S. (2009) On-line brush and spray washers to lower numbers of *Campylobacter* and *Escherichia coli* and presence of *Salmonella* on broiler carcasses during processing. *Journal of Applied Poultry Research* **18**, 74-78.

Bland,J.M. and Altman,D.G. (1986) Statistical Methods for Assessing Agreement Between Two Methods of Clinical Measurement. *Lancet* **1**, 307-310.

Bouwknegt,M., van de Giessen,A.W., Dam-Deisz,W.D.C., Havelaar,A.H., Nagelkerke,N.J.D. and Henken,A.M. (2004) Risk factors for the presence of *Campylobacter* spp. in Dutch broiler flocks. *Preventive Veterinary Medicine* **62**, 35-49.

Chinivasagam,H.N., Tran,T., Maddock,L., Gale,A. and Blackall,P.J. (2009) Mechanically Ventilated Broiler Sheds: a Possible Source of Aerosolized *Salmonella*, *Campylobacter*, and *Escherichia coli*. *Applied and Environmental Microbiology* **75**, 7417-7425.

Chowdhury,S., Themudo,G.E., Sandberg,M. and Ersboll,A.K. (2013) Spatio-temporal patterns of *Campylobacter* colonization in Danish broilers. *Epidemiology and Infection* **141**, 997-1008.

Cowell,N.D. and Morisett,M.D. (1969) Microbiological Techniques - Some Statistical Aspects. *Journal of the Science of Food and Agriculture* **20**, 573-&.

de Albano,F.M. and ten Caten,C.S. (2014) Proficiency tests for laboratories: a systematic review. *Accreditation and Quality Assurance* **19**, 245-257.

Dumont,F., Marechal,P.A. and Gervais,P. (2004) Cell size and water permeability as determining factors for cell viability after freezing at different cooling rates. *Applied and Environmental Microbiology* **70**, 268-272.

Friedrich,A., Marshall,J.C., Biggs,P.J., Midwinter,A.C. and French,N.P. (2016) Seasonality of *Campylobacter jejuni* isolates associated with human campylobacteriosis in the Manawatu region, New Zealand. *Epidemiology and Infection* **144**, 820-828.

Gibbens,J.C., Pascoe,S.J.S., Evans,S.J., Davies,R.H. and Sayers,A.R. (2001) A trial of biosecurity as a means to control *Campylobacter* infection of broiler chickens. *Preventive Veterinary Medicine* **48**, 85-99.

Harrison,D., Corry,J.E.L., Tchorzewska,M.A., Morris,V.K. and Hutchison,M.L. (2013) Freezing as an intervention to reduce the numbers of campylobacters isolated from chicken livers. *Letters in Applied Microbiology* **57**, 206-213.

Haughton,P.N., Lyng,J., Cronin,D., Fanning,S. and Whyte,P. (2012) Effect of crust freezing applied alone and in combination with ultraviolet light on the survival of *Campylobacter* on raw chicken. *Food Microbiology* **32**, 147-151.

Hazeleger,W.C., Bolder,N.M., Beumer,R.R. and Jacobs-Reitsma,W.F. (2008) Darkling Beetles (Alphitobius diaperinus) and Their Larvae as Potential Vectors for the Transfer of *Campylobacter jejuni* and *Salmonella enterica* Serovar Paratyphi B Variant Java between Successive Broiler Flocks. *Applied and Environmental Microbiology* **74**, 6887-6891.

Hinton,A., Cason,J.A. and Ingram,K.D. (2004) Tracking spoilage bacteria in commercial poultry processing and refrigerated storage of poultry carcasses. *International Journal of Food Microbiology* **91**, 155-165.

Hog,B.B., Sommer,H.M., Larsen,L.S., Sorensena,A.I.V., David,B., Hofshagen,M. and Rosenquist,H. (2016) Farm specific risk factors for *Campylobacter* colonisation in Danish and Norwegian broilers. *Preventive Veterinary Medicine* **130**, 137-145.

Hue,O., Le Bouquin,S., Laisney,M.J., Allain,V., Lalande,F., Petetin,I., Rouxel,S., Quesne,S., Gloaguen,P.Y., Picherot,M., Santolini,J., Salvat,G., Bougeard,S. and Chemaly,M. (2010) Prevalence of and risk factors for *Campylobacter* spp. contamination of broiler chicken carcasses at the slaughterhouse. *FOOD MICROBIOLOGY* **27**, 992-999.

Hutchison,M.L., Walters,L.D., Avery,S.M., Reid,C.A., Wilson,D., Howell,M., Johnston,A.M. and Buncic,S. (2005) A comparison of wet-dry swabbing and excision sampling methods for microbiological testing of bovine, porcine, and ovine carcasses at red meat slaughterhouses. *Journal of Food Protection* **68**, 2155-2162.

Hutchison,M.L., Walters,L.D., Allen,V.M., Mead,G.C. and Howell,M. (2006a) Measurement of *Campylobacter* numbers on carcasses in British poultry slaughterhouses. *Journal of Food Protection* **69**, 421-424.

Hutchison,M.L., Walters,L.D., Mead,G.C., Howell,M. and Allen,V.N. (2006b) An assessment of sampling methods and microbiological hygiene indicators for process verification in poultry slaughterhouses. *Journal of Food Protection* **69**, 145-153.

International Organization for Standardization (2006) CD 10272:2. Microbiology of food and animal feeding stuffs - Horizontal method for detection and enumeration of *Campylobacter* spp. -Part 2: Colony-count technique. Geneva: International Organization for Standardization.

Jackson,R.W., Osborne,K., Barnes,G., Jolliff,C., Zamani,D., Roll,B., Stillings,A., Herzog,D., Cannon,S. and Loveland,S. (2000) Multiregional evaluation of the SimPlate heterotrophic plate count method compared to the standard plate count agar pour plate method in water. *Applied and Environmental Microbiology* **66**, 453-454.

Japp,A.K., Bicho,C.D. and da Silva,A.V.F. (2010) Importance and measures of control for Alphitobius diaperinus in poultry houses. *Ciencia Rural* **40**, 1668-1673.

Jonsson,M.E., Chriel,M., Norstrom,M. and Hofshagen,M. (2012) Effect of climate and farm environment on *Campylobacter* spp. colonisation in Norwegian broiler flocks. *Preventive Veterinary Medicine* **107**, 95-104.

Jørgensen, F., Bailey, R., Williams, S., Henderson, P., Wareing, D. R., Bolton, F. J., Frost, J.A., Ward, L., and Humphrey T. J. (2002). Prevalence and numbers of *Salmonella* and *Campylobacter* spp. on raw, whole chickens in relation to sampling methods. *Int J Food Microbiol*. **76**,151-64

Madden,R.H., Ball,H., Hutchison,M., Young,F. and Taylor,M. (2014) A quantitative comparison of sample matrices for the detection of *Campylobacter* spp. in broiler houses. *Romanian Biotechnological Letters* **19**, 9785-9791.

McDowell,S.W.J., MenzieS,F.D., McBride,S.H., Oza,A., McKenna,J.P., Gordon,A.W. and Neillab,S.D. (2008) *Campylobacter* spp. in conventional broiler flocks in Northern Ireland: Epidemiology and risk factors. *Preventive Veterinary Medicine* **84**, 261-276.

Meunier,M., Guyard-Nicodeme,M., Dory,D. and Chemaly,M. (2016) Control strategies against *Campylobacter* at the poultry production level: biosecurity measures, feed additives and vaccination. *Journal of Applied Microbiology* **120**, 1139-1173.

Moran, L., Scates, P. J. and Madden, R. H. (2009). Prevalence of *Campylobacter* spp. in raw retail poultry on sale in Northern Ireland. *Journal of Food Protection*. **72**, 1830-1835

Nuttal,D.L., Goldstein,H., Prosser,R. and Rashbash,J. (1989) Differential school effectiveness. *International Journal of Education Research* **13**, 769-776.

Pitkanen,T. (2013) Review of *Campylobacter* spp. in drinking and environmental waters. *Journal of Microbiological Methods* **95**, 39-47.

Pourabedin,M. and Zhao,X. (2015) Prebiotics and gut microbiota in chickens. *Fems Microbiology Letters* **362**.

Roberts,T.A., Macfie,H.J.H. and Hudson,W.R. (1980) The Effect of Incubation-Temperature and Site of Sampling on Assessment of the Numbers of Bacteria on Red Meat Carcasses at Commercial Abattoirs. *Journal of Hygiene* **85**, 371-380.

Russell,S.M. and Axtell,S.P. (2005) Monochloramine versus sodium hypochlorite as antimicrobial agents for reducing populations of bacteria on broiler chicken carcasses. *Journal of Food Protection* **68**, 758-763.

Saathoff,E., Olsen,A., Magnussen,P., Kvalsvig,J.D., Becker,W. and Appleton,C.C. (2004) Patterns of *Schistosoma haematobium* infection, impact of praziquantel treatment and re-infection after treatment in a cohort of schoolchildren from rural KwaZulu-Natal/South Africa. *Bmc Infectious Diseases* **4**.

Sahin,O., Kassem,I.I., Shen,Z.Q., Lin,J., Rajashekara,G. and Zhang,Q.J. (2015) *Campylobacter* in Poultry: Ecology and Potential Interventions. *Avian Diseases* **59**, 185-200.

Skirrow, M. B.(1987) A demographic survey of *Campylobacter*, *Salmonella* and *Shigella* infections in England. A Public Health Laboratory Service Survey. *Epidemiol Infect.* **99,**647-57.

Sommer,H.M., Heuer,O.E., Sorensen,A.I.V. and Madsen,M. (2013) Analysis of factors important for the occurrence of *Campylobacter* in Danish broiler flocks. *Preventive Veterinary Medicine* **111**, 100-111.

Stead,D. and Park,S.F. (2000) Roles of Fe superoxide dismutase and catalase in resistance of *Campylobacter coli* to freeze-thaw stress. *Applied and Environmental Microbiology* **66**, 3110-3112.

Takahashi,R., Shahada,F., Chuma,T. and Okamoto,K. (2006) Analysis of *Campylobacter* spp. contamination in broilers from the farm to the final meat cuts by using restriction fragment length polymorphism of the polymerase chain reaction products. *International Journal of Food Microbiology* **110**, 240-245.

Tam, C. C., O'Brien, S. J., Tompkins, D. S., Bolton, F. J., Berry, L., Dodds, J., Choudhury, D., Halstead, F., Iturriza-Gómara, M., Mather, K., Rait, G., Ridge, A., Rodrigues, L. C., Wain, J., Wood, B., Gray, J. J. & the IID2 Study Executive Committee (2012) Changes in causes of acute gastroenteritis in the United Kingdom over 15 years: microbiologic findings from two prospective, population-based studies of infectious intestinal disease. *Clin. Infect. Dis.* **54,** 1275-1286.

Thompson,M., Ellison,S.L.R. and Wood,R. (2006) The International Harmonized Protocol for the proficiency testing of analytical chemistry laboratories (IUPAC technical report). *Pure and Applied Chemistry* **78**, 145-196.

Toner,M., Cravalho,E.G. and Karel,M. (1990) Thermodynamics and Kinetics of Intracellular Ice Formation During Freezing of Biological Cells. *Journal of Applied Physics* **67**, 1582-1593.

Willfor,S., Nisula,L., Hemming,J., Reunanen,M. and Holmbom,B. (2004) Bioactive phenolic substances in industrially important tree species. Part 1: Knots and stemwood of different spruce species. *Holzforschung* **58**, 335-344.

Wood, R., Nilson, A. and Wallen, H. (1998) Appendix I. Procedure for the estimation and expression of measurement of uncertainty in chemical analysis developed by the Nordic committee on food analyses. In *Quality in the food analysis laboratory* ed. Elton,P.S. pp. 135-147. Cambridge: The Royal Society of Chemistry Information Services.

Yun,J., Greiner,M., Holler,C., Messelhausser,U., Rampp,A. and Klein,G. (2016) Association between the ambient temperature and the occurrence of human *Salmonella* and *Campylobacter* infections. *Scientific Reports* **6**.