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Seasonal and within-herd variability of *E. coli* concentrations in fresh dairy faeces

David M Oliver

Biological and Environmental Sciences
School of Natural Sciences
University of Stirling
Stirling
FK9 4LA
UK

Corresponding author: david.oliver@stir.ac.uk, Biological & Environmental Sciences, School of Natural Sciences, University of Stirling, Scotland, UK. Tel: +44 1786 467846; Fax: +44 1786 467843.

Significance and impact of the study

This study provides a comprehensive temporal dataset of faecal indicator organism (FIO) counts (both *E. coli* and other coliforms) in fresh dairy faeces for Scotland. Such faecal audits for the UK are scarce which is surprising given that livestock constitute one of the largest agricultural sources of diffuse microbial pollution of surface waters and contributors to poor bathing water quality. Such FIO concentration data (and evaluation of variability across seasonal, within-herd, and year-on-year counts) in fresh faeces is a fundamental precursor to the robust parameterization of models that aim to predict the fate and transfer of both FIOs and pathogens in agricultural catchments.

Abstract

The aim of this study was to determine concentrations of culturable faecal indicator organisms (FIOs) in freshly excreted dairy faeces and assess seasonal, within-herd and year-on-year variability in counts. Such values are essential in order to provide input parameters and associated uncertainty bounds for empirical models designed to determine the burden of FIOs on pasture. A longitudinal faecal analysis survey (n=80) was conducted at a conventional dairy farm in central Scotland over a two-year period. The analysis quantified counts of *Escherichia coli* and other non-*E. coli* coliforms and compared the concentrations of these FIO groups across contrasting seasons. The overall mean concentration of *E. coli* was 6.63 and 6.58 $\log_{10}$ CFU g^{-1} dry weight in 2012 and 2013, respectively. However, concentrations of *E. coli* in faecal pats on each seasonal sampling event were highly variable and spanned several orders of magnitude on all occasions. Concentrations of *E. coli* in faeces excreted in winter were found to be lower than those excreted in all other seasons in 2012, though patterns of seasonal shedding were not consistent in observations the

following year highlighting additional sources of uncertainty in FIO loading to land from dairy herds.

Keywords: agriculture; cattle; diffuse pollution; *Escherichia coli*; faecal coliforms; livestock faeces; modelling

Introduction

Escherichia coli are commonly used as a faecal indicator organism (FIO) by environment protection agencies throughout the world. While the presence (or absence) of FIOs does not confirm the presence (or absence) of a pathogen (Wu et al., 2011) their detection in environmental matrices is indicative of pollution originating from a faecal source (Blaustein et al., 2013). These bacteria, which make up the majority of the faecal coliform (FC) group, can be released into the wider environment following livestock defecation and/or manure and slurry applications to land, and via wastewater releases from sewage treatment works or septic tanks (Kay et al., 2008; Chadwick et al., 2009). In catchments dominated by livestock agriculture the accumulation of FIOs on pasture is a dynamic function of livestock numbers, their faecal excretion and bacterial shedding capacity, and bacterial die-off rates as determined by environmental drivers such as temperature and intensity of UV radiation (Oliver et al., 2010a).

The concentrations of *E. coli* found in freshly excreted livestock faeces can vary by several orders of magnitude (Cox et al, 2005; Muirhead et al., 2006; Ferguson et al., 2009). The drivers that contribute to this variation have been suggested to include diet, animal age, and livestock type, among other factors (Russell et al., 2000; Moriarty et al., 2008; Oliver et al., 2010b). This variability in shedding is not only linked to large scale faecal surveys across multiple farms, regions or countries; some

104 studies have reported large variation from within a single herd (e.g. Donnison et al.,
105 2008).
106
107 This variation of *E. coli* shedding poses a significant challenge for the development of
108 modelling approaches to predict the fate and transfer of microbial contaminants
109 through agricultural catchments (Oliver et al., 2012). The growing requirement for the
110 design of 'programmes of measures' by Article 11 of the Water Framework Directive
111 (WFD), to prevent impairment of 'protected areas' (i.e. including bathing and shellfish
112 harvesting waters), is generating an imperative for the development of modelling
113 capacity. This is needed in order to differentiate specific (spatial) effects of land
114 management practices when combined with catchment responses to hydrological
115 drivers at relevant timescales. However, such models need to account for the source
116 strength of faecal reservoirs attributed to different livestock types and while the
117 current evidence-base is growing it remains far from satisfactory. From a UK
118 perspective there is an urgent need for an inventory of *E. coli* concentrations
119 associated with a suite of livestock types for different regions where livestock farming
120 dominates. However, rather than a comprehensive evidence-base that captures
121 variability of regional *E. coli* counts, there are few studies that provide useful
122 information (e.g. Avery et al., 2004; Hodgson et al., 2009), and arguably not enough
123 for widespread spatial and temporal modelling of FIO accumulation on pasture. This
124 situation is not unique to the UK. For example, Moriarty et al (2008) highlighted the
125 dearth of published counts of bacterial indicators in fresh livestock faeces across
126 New Zealand and in response undertook a faecal survey across four farm
127 environments spanning the North and South Islands. With limited national data,
128 those who aim to develop microbial fate and transfer models must either undertake
129 faecal surveys as per Moriarty et al (2008) or instead draw on microbial counts

published in the wider international literature. Of course, these latter values may not be particularly relevant to local conditions.

Clearly a national inventory of typical FIO counts would take time to evolve and necessitate significant effort to develop. However, the need for better quality information and a robust empirical evidence-base on FIO concentrations for different geographical areas, livestock types and seasons, is fundamental for underpinning our understanding of diffuse microbial pollution from agriculture (and informing mitigation strategies to reduce its impact). Similar issues have been raised with regard to knowledge of the likely FIO concentrations in raw sewage and treated effluents. Kay et al (2008) identified that few empirical data had been published in the peer reviewed literature for these effluent types and provided a summary of FIO concentrations determined from 162 sewage discharge sites across the UK and Jersey, and stressed the importance of this data for prioritising suitable management approaches to water quality protection.

Without a thorough understanding of how the burden of FIOs on pasture varies through an annual cycle (and its susceptibility to vary year-on-year) our landscape-level models of microbial fate and transfer are immediately disadvantaged in terms of their predictive capability. This study was therefore designed to contribute important information on FIO concentrations in dairy faeces – one of the key sources of diffuse microbial pollution from agricultural landscapes. The aim of the study was to quantify seasonal, within-herd and year-on-year variability of FIO (both *E. coli* and other coliform) concentrations in freshly excreted dairy faeces from a typical farm enterprise in central Scotland.

Results and Discussion

This study provides a significant dataset relating to the potential for *E. coli* and coliform loading to agricultural land by dairy cattle in central Scotland. By following the same herd over a two year period the study has documented the temporal profile of this variability and highlighted: (i) seasonal impacts on the magnitude of *E. coli* excreted in fresh faeces of dairy cows; and (ii) how seasonal shedding patterns can fluctuate over successive years. The importance of FIO concentration data in fresh faeces cannot be understated as it provides information that is crucial for the parameterization of models that aim to predict pathogen and FIO fate and transfer in agricultural catchments (Moriarty et al., 2008; Oladeinde et al., 2014). All microbial counts are presented on a fresh and dry weight basis to enable a wider comparison across the literature. All *E. coli* counts, and all but the spring 2012 combined coliform counts were confirmed as being log-normally distributed (see Table 1 for normality assessment on the fresh weight counts using the Shapiro-Wilk test).

All method blanks were negative for FIOs indicating no cross contamination during sample processing. The mean concentration of *E. coli* determined in fresh dairy faeces for all samples collected across all seasons was found to be 6.63 and 6.58 $\log_{10}$ CFU g^{-1} dry weight for 2012 and 2013, respectively. Interestingly, Martinez et al (2013) reported that the average *E. coli* concentration in fresh faecal material (based on combined data from six international studies) equated to 6.5 $\log_{10}$ CFU g^{-1}, which is close to the average values recorded in both years of this study. A series of boxplots are presented in Figure 1 to highlight the contrasting variability in concentrations of *E. coli* excreted in dairy faeces across different seasons over the two-year period, with magnitudes represented on a dry weight basis. Table 1 shows the counts (mean, min and max) for both *E. coli* and a combined coliform count (*E. coli* plus all other non-*E. coli* coliforms) on a wet weight basis for comparison. There was little difference in the representation of *E. coli* and combined coliform counts and

184 so the statistical analysis focused on the *E. coli* counts for brevity. With all data
185 combined, a two-way ANOVA identified a significant difference between the counts
186 determined for different seasons (P <0.001) but not for the overall mean of *E. coli*
187 counts observed in successive years. In 2012 the counts associated with winter
188 faecal deposits (mean of 5.72 $\log_{10}$ CFU g^{-1} dry weight) were significantly lower (P
189 <0.05) than those determined in spring, summer and autumn faecal deposits (mean
190 of 6.90, 6.79 and 7.10 $\log_{10}$ CFU g^{-1} dry weight, respectively). While the overall mean
191 of *E. coli* concentrations did not differ between 2012 and 2013 it was revealed that
192 seasonal differences in 2013 did not mirror those observed in 2012. In 2013 autumn
193 and winter faecal deposits were both found to have significantly lower counts of *E.*
194 *coli* (mean of 6.25 and 6.16 $\log_{10}$ CFU g^{-1} dry weight, respectively) relative to summer
195 (P <0.05; mean count of 7.37 $\log_{10}$ CFU g^{-1} dry weight) but were not significantly
196 lower than those recorded in spring (see Fig 1).
197
198 A number of studies have been published that report, to varying extents, on
199 concentrations of FIOs in fresh cattle faeces in New Zealand (Moriarty et al., 2008;
200 Sinton et al; Donnison et al., 2008; Muirhead and Littlejohn, 2009), the US (Weaver
201 et al., 2005; van Kessel et al., 2007; Soupir et al., 2008), Canada (Meays et al.,
202 2005), Australia (Cox et al., 2005) and the UK (Avery et al., 2004; Hodgson et al.,
203 2009). All of these studies report variability in concentrations of FIOs in fresh faeces,
204 often in excess of at least 1 order of magnitude and this result is consistent with the
205 data reported in this current study. There are contrasting observations evident in the
206 international literature with studies reporting peak concentrations of FIOs associated
207 with different seasons (e.g. Sinton et al., 2007; Moriarty et al., 2008; Muirhead and
208 Littlejohn, 2009). Differences in observations at a national level may reflect variations
209 in dietary supplements available to livestock during housing periods (Russell et al.,
210 2000) or anxiety levels of livestock associated with management regimes (Bach et

al., 2004). Studies also vary in their use of 'naturally' deposited cowpats versus artificially homogenized fresh faecal material crafted into replicate cowpats and this may also play a role in the observed variability. For example, recent research by Martinez et al. (2013) analyzed data on FIOs in fresh cowpats obtained from a number of studies at different locations across the world and identified that repacked cowpats had a significantly higher *E. coli* content than naturally intact cowpats. The same authors also reported that, using this combined international dataset, artificial repacked cowpats exhibited relatively small differences in initial concentrations of *E. coli* in cowpats across different seasons compared to seasonal differences observed in their naturally intact counterparts.

The results of the current study confirm that in 2012, autumn > spring > summer > winter with regard to the concentrations of *E. coli* detected in fresh dairy faeces on the monitored farm in Scotland. For 2013 this ranking shifted to summer > spring > autumn > winter. Two observations are clear from an inspection of these seasonal rankings: (i) patterns and seasonal peaks of *E. coli* shedding by dairy cattle are not consistent year on year; but (ii) winter does appear to be somewhat consistent in generating dairy faeces with substantially lower *E. coli* counts relative to other seasons (for a two year cycle at least). The apparent shifts in ranking of seasonal *E. coli* shedding for this study in Scotland may reflect local conditions linked to diet and management that were indirectly impacted by weather conditions. While climatic variables (e.g. temperature and rainfall) cannot be held directly accountable for fresh *E. coli* concentrations in faeces, because the cells will be held within the animal gut and gastrointestinal tract at 37^{o}C prior to excretion, such environmental factors might influence on-farm management decisions (e.g. changes in grazing management that necessitate a shift in livestock diet) that may then have consequential impacts on *E. coli* shedding by cattle.

238

239 For example in this study, during 2012, dairy cattle were put out to pasture for
240 grazing at the end of April (i.e. mid-spring) but were re-housed relatively early (i.e.
241 July; mid-summer) because of exceptionally wet conditions that rendered grazing
242 activity detrimental to soil and pasture quality. Indeed, summer 2012 ranked as the
243 second wettest in the UK since records began in 1910 and 121% of the 1961 to 1990
244 UK average rainfall was recorded during 2012 (MET Office, 2012). The cattle were
245 reintroduced to pasture later in the summer of 2012 and grazed until early
246 September before being rehoused again for autumn and winter. In contrast, the 2013
247 grazing regime was more straightforward with cattle grazing from the end of April
248 through to the beginning of October. The diet of the cows was necessarily different
249 during the contrasting grazing and housed periods. During grazing, the dietary intake
250 of cattle was predominantly perennial ryegrass *Lolium perenne* and this was
251 supplemented with dairy cake (an 18% protein mix containing wheat and distiller's
252 grains) during milking. During the housed period, their diet consisted mainly of silage
253 combined with distiller's grains, brewer's barley and molasses, and again this was
254 supplemented with dairy cake (at an increased 20% protein mix) during milking.
255 Given that the winter period in both years resulted in the lowest counts of *E. coli* in
256 fresh dairy faeces it is possible that the housed diet of predominantly silage helped to
257 reduce generic *E. coli* levels excreted, or at least rendered a proportion as viable-but-
258 non-culturable. In a comparison of faeces excreted from silage- and pasture-fed
259 cows the concentrations of *E. coli* have been shown to be lower (by ~ 1 order of
260 magnitude) and more variable for those given a silage diet (Donnison et al., 2008).
261 The fermentation process typical of silage production results in the generation of
262 acids, such as lactic acid, that preserves the silage and the resulting reduction in
263 rumen pH can reduce naturally occurring *E. coli* that do not grow well at low pH
264 values (Russell et al., 2000). In addition, Donnison et al. (2008) hypothesise that the

higher counts associated with pasture-fed diet may reflect a continuous ingestion of FIOs from faecally contaminated pasture. Interestingly, the 2012 summer FIO concentrations ranked lower relative to their 2013 ranking and this might reflect the removal of the cows from a pasture-fed diet to one of silage during their temporary summer housing because of the exceptionally wet weather in 2012 which was not repeated in 2013.

Statistical analysis using a paired *t*-test on duplicate samples taken from 40 cowpats across all seasons recorded no significant difference (P =0.58) in *E. coli* counts. This suggests that faecal excretion by dairy cattle is effective in homogenizing *E. coli* populations in the faecal matrix and supports the hypothesis that FIOs are thoroughly mixed following faecal passage through the ruminant digestive system and gut. This contrasts with observations for specific pathogens such as *E. coli* O157 (Robinson et al., 2005) where cells remain heterogeneously distributed within the faeces. The mean %DM of fresh dairy faeces for all samples collected across all seasons was 13.83% and 13.22% for 2012 and 2013, respectively. The underlying dry matter content of all faecal deposits is presented in Table 2 (mean, median and range) and the variability in % dry matter is shown in Figure 2 for all seasons across both years. For all data combined, two-way ANOVA identified a significant difference between the % dry matter determined in different seasons (P <0.001) but there was no significant difference for the overall mean of %DM recorded across all seasons in 2012 versus 2013. In 2012 the faecal deposits excreted in summer had a significantly lower (P <0.05) DM than those excreted in all other seasons. In 2013 differences in faecal pat DM across seasons were more complex (see Fig 2) although the summer deposits still retained a significantly lower %DM relative to all other seasons (P <0.05) despite accommodating the largest range of %DM recorded across both years of the study. No correlation between %DM content and FIO

concentrations in fresh dairy faeces was observed. Moriarty et al (2008) observed a consistent increase in total solid content of fresh dairy faeces from spring to winter and found the winter total solids content to be approximately double that observed in faeces excreted in spring. In our study this pattern was not observed and for both 2012 and 2013 the faeces excreted in summer contained the lowest dry matter content. The lower DM in summer is probably a consequence of diet with pasture forming the predominant source of feed. The higher DM in winter through spring is likely to reflect the diet shift from pasture to silage.

The empirical data reported in this study has highlighted considerable variability in *E. coli* and coliform concentrations and their susceptibility to change seasonally, both between and within annual cycles. This has important implications for modeling approaches that choose to use a single parameter for an *E. coli* concentration typical of dairy faeces (and most probably other faeces associated with other livestock types too) without considering (i) within-herd variation in shedding and (ii) how this seasonal shift in variability might impact on predictions of FIO risk dynamics over time for a given area. Studies such as the one presented here need to be repeated across different regions of the UK to build up a better profile of how FIO concentrations vary spatially and in time. Developing an inventory of microbial magnitudes in fresh faeces and improving our understanding of their scope to vary is an important factor to build into modeling approaches and to communicate to catchment stakeholders interested in microbial risks associated with land and water. A concerted effort is essential in order to consolidate this important evidence base so that uncertainties surrounding FIO concentrations can not only be acknowledged but also used to improve the quality of models of microbial fate and transfer in catchments.

Materials and Methods

Sample collection

Ten fresh dairy cowpats were collected on eight sampling occasions over a two year period. Samples were collected in March, June, September and December of 2012 and 2013 and represented faeces excreted at the start of each season (spring, summer, autumn and winter, in the northern hemisphere). The ten cowpats served as replicate samples and were collected from ten different cows on each sampling occasion. Thus, a total of 80 cowpats were collected throughout the study period. The cowpats were collected from a single conventional 165 ha dairy farm in Stirlingshire, Scotland. The dairy herd totaled 80 head of cattle, was normally housed from October through to the end of March, and produced an average of 8000 litres of milk per year per cow. All cowpats were collected within 30 minutes of excretion. Fresh samples were collected from a covered holding-barn that was used during the transfer of dairy cows to the parlour for morning milking. This barn was scraped clean twice daily and so all cowpats collected were assured to be fresh deposits.

All cowpats were collected from Holstein Friesians used for milk production and were sampled and analysed for *Escherichia coli,* coliforms, and dry matter (DM) content. Microbial analysis was initiated within one hour of samples being collected. Approximately 15g of faeces was randomly sampled from each cowpat using a sterile spatula (70% IMS, rinsed with sterile water) and placed into sterile 50 mL centrifuge tubes. Samples were assumed to be well mixed and homogeneous following faecal passage through the ruminant digestive system and gut. However, for 50% of the cowpats, a duplicate random sample was taken from the faeces to investigate whether the sampling approach could potentially impact on recorded FIO counts because of uneven distribution of cells within the faecal matrix (i.e. spatial bias in counts). Only the original sample was used in the wider analysis reported in

this study but the duplicate sample served an important purpose as a subcomponent of this faecal survey, as described.

Sample analysis

One gram of fresh faeces was used for microbial analysis and the remainder was used to determine the gravimetric water content by drying at 105ºC for 24 h (until constant mass) and weighing the residual. For microbial analysis, one gram of faeces was transferred to 9 mL of sterile phosphate buffered saline (PBS) and then thoroughly mixed using an orbital shaker (160 rpm for 60 minutes at ambient temperature) to disperse cells from the faecal matrix. Further serial 1:10 dilutions were then made as appropriate to ensure capture of between 20 to 200 colony forming units (CFU) once the sample had been transferred to an agar growth medium. To get to this stage, 1mL of each serially-diluted sample was washed through a filtration unit (Sartorius, Germany) with ~20 mL of sterile PBS. Membrane filters of 0.45 micron pore size (Sartorius, Germany) were aseptically transferred to Membrane Lactose Glucuronide Agar (MLGA) (Oxoid, Basingstoke, UK) and incubated inverted at 37ºC (±0.2ºC) for 18–24 h for the determination of presumptive *E. coli* and other coliform colonies. Equipment was flame sterilized between samples and method blanks (i.e. sterile PBS) used to confirm the sterilization procedure. The limit of detection was 100 CFU per g fresh weight faeces.

Statistical Analysis

All counts were transformed to $\log_{10}$ CFU and distributions of *E. coli* were log normally distributed as determined using the Shapiro-Wilk goodness of fit test. Treatment (season, year) differences in *E. coli* and %DM were compared by two-way analysis of variance (ANOVA) for all data combined. One-way ANOVA was used to test for differences across individual years and Tukey multiple comparison tests

applied (Minitab 12.0 software, Minitab Inc., PA, USA). A paired *t*-test was used to determine whether there was any significant difference between repeated sampling of different sub-components of the same cowpat.

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Conflicts of interest

No conflict of interest declared.

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476 **List of Figures**

477 **Fig 1:** Seasonal, within-herd and year-on-year variability of *E. coli* concentrations in
478 fresh dairy faeces. Boxplots with different letter codes differ significantly from one
479 another (2012 data: one-way ANOVA, P <0.001; Tukey multiple comparison test, P <
480 0.05 & 2013 data: one-way ANOVA , P = 0.016; Tukey multiple comparison test, P <
481 0.05). Centre horizontal dash, box and whiskers represent median, inter-quartile
482 range and upper & lower limits, respectively. Values are the mean of 10 replicates.
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484

485 **Fig 2:** Seasonal, within-herd and year-on-year variability of % dry matter content in
486 fresh dairy faeces. Boxplots with different letter codes differ significantly from one
487 another (2012 data: one-way ANOVA, P <0.001; Tukey multiple comparison test, P <
488 0.05 & 2013 data: one-way ANOVA , P <0.001; Tukey multiple comparison test, P <
489 0.05). Centre horizontal dash, box and whiskers represent median, inter-quartile
490 range and upper & lower limits, respectively. * signifies an extreme value. Values are
491 the mean of 10 replicates.
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502 **Table 1**: Summary of *E. coli* and combined coliform counts (*E. coli* + other non-*E. coli* coliform bacteria) on a wet weight basis. All counts
503 derived from 10 cowpats per sampling event.

Sampling date	***E. coli* (log_{10} CFU g^{-1} wet weight)**			**Combined coliforms (log_{10} CFU g^{-1} wet weight)**			***E. coli***	**All coliforms**
	Mean	**Min**	**Max**	**Mean**	**Min**	**Max**	***P* value (Sig different to Lognormal distribution?)**	***P* value (Sig different to Lognormal distribution?)**
Spring 2012	6.07	5.22	7.47	6.27	5.49	7.82	>0.10	<0.01
Summer 2012	5.87	5.03	6.56	6.89	6.06	7.61	>0.10	>0.15
Autumn 2012	6.24	5.38	6.97	6.28	5.40	7.00	>0.10	>0.15
Winter 2012	4.87	3.54	6.40	4.90	3.54	6.52	>0.08	0.11
Spring 2013	5.69	3.84	7.03	5.69	3.84	7.03	>0.10	>0.15
Summer 2013	6.89	6.02	8.44	6.91	6.04	8.44	>0.10	>0.15
Autumn 2013	5.34	4.54	6.84	5.38	4.55	6.84	>0.10	>0.15
Winter 2013	5.74	4.51	7.12	5.81	4.83	7.12	>0.10	>0.15

504 **Table 2:** Dry matter (DM) content of dairy faeces collected throughout the 2 year
505 study. All counts derived from 10 cowpats per sampling event.
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Sampling date	Mean % DM	Median % DM	Range of % DM (magnitude)
Spring 2012	15.19	15.16	13.96 – 16.99 (3.04)
Summer 2012	11.95	11.82	9.72 – 14.52 (4.80)
Autumn 2012	14.07	13.93	11.97 – 18.31 (6.33)
Winter 2012	14.10	13.90	13.03 – 15.26 (2.23)
Spring 2013	13.92	13.86	12.34 – 15.55 (3.21)
Summer 2013	11.85	11.54	9.25 – 17.44 (8.19)
Autumn 2013	12.35	12.21	9.89 – 14.25 (4.36)
Winter 2013	14.76	14.35	13.28 – 17.42 (4.14)

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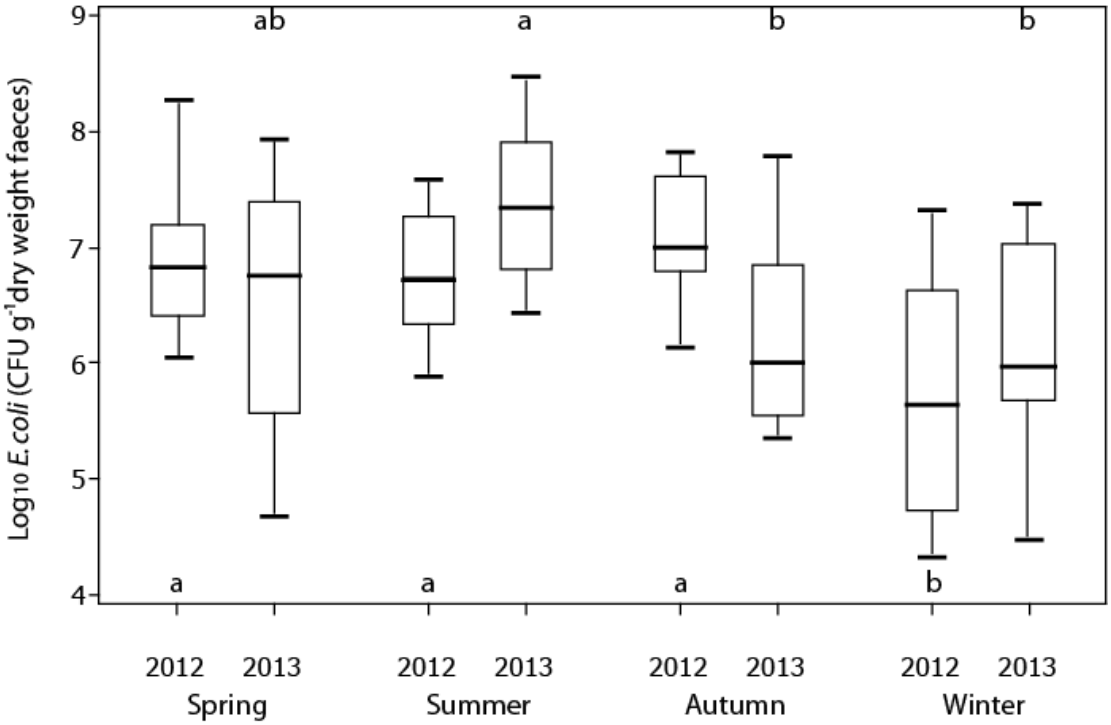

Fig 1: Seasonal, within-herd and year-on-year variability of *E. coli* concentrations in fresh dairy faeces. Boxplots with different letter codes differ significantly from one another (2012 data: one-way ANOVA, P <0.001; Tukey multiple comparison test, P < 0.05 & 2013 data: one-way ANOVA , P = 0.016; Tukey multiple comparison test, P < 0.05). Centre horizontal dash, box and whiskers represent median, inter-quartile range and upper & lower limits, respectively. Values are the mean of 10 replicates.

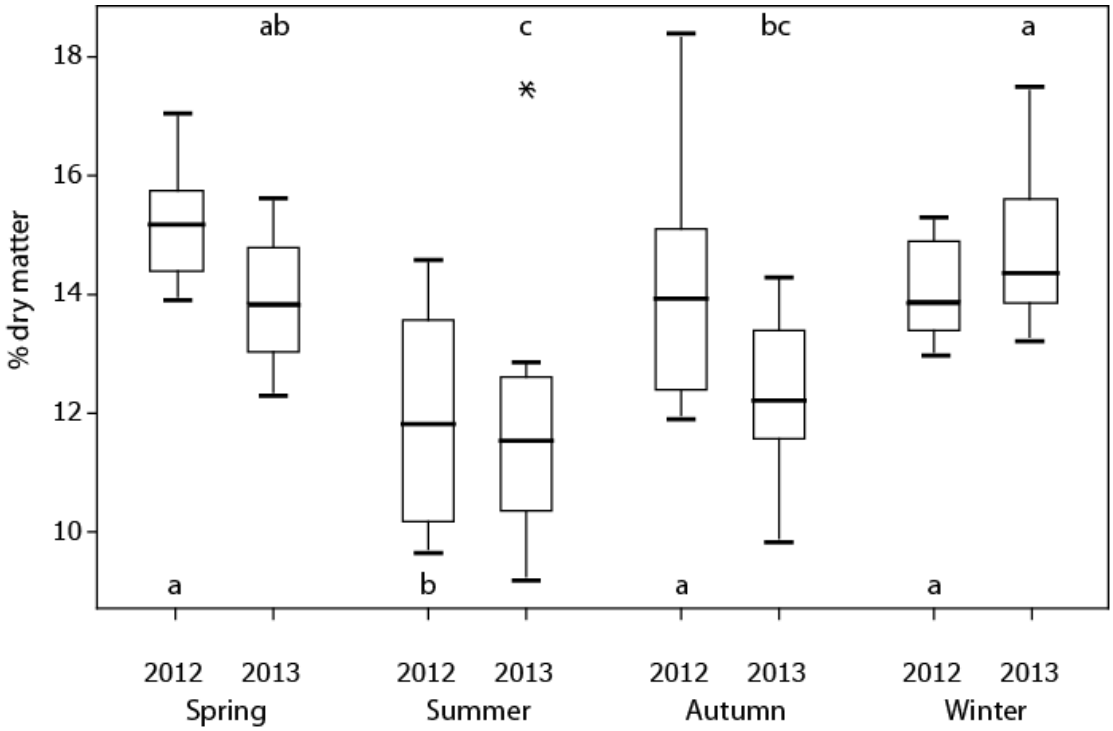

Fig 2: Seasonal, within-herd and year-on-year variability of % dry matter content in fresh dairy faeces. Boxplots with different letter codes differ significantly from one another (2012 data: one-way ANOVA, P <0.001; Tukey multiple comparison test, P < 0.05 & 2013 data: one-way ANOVA , P <0.001; Tukey multiple comparison test, P < 0.05). Centre horizontal dash, box and whiskers represent median, inter-quartile range and upper & lower limits, respectively. * signifies an extreme value. Values are the mean of 10 replicates.