The occurrence of *Campylobacter* spp., *Listeria monocytogenes* and Shiga toxin-producing *Escherichia coli* in Norwegian dairy cattle farms; a comparison between free stall and tie stall housing systems

Lene Idland[1], Erik G. Granquist[2], Marina Aspholm[1], Toril Lindbäck[1*]

[1]Department of Paraclinical Sciences, Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Ås, Norway

[2]Department of Production Animal Clinical Sciences, Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Ås, Norway

Abbreviated headline: Pathogens in Norwegian in raw milk

* Correspondence:

Toril Lindbäck

toril.lindback@nmbu.no

Abstract

Aims

This study explores how different dairy farm operating systems influence the occurrence of zoonotic bacteria in raw milk.

Methods and Results

Samples from bulk tank milk, milk filters, feces, feed, teats and teat milk were collected from eleven farms with loose housing and seven with tie-stall housing every second month over a period of 11 months and analyzed for the presence of *Campylobacter* spp., *L. monocytogenes* and STEC. *Campylobacter* spp., *L. monocytogenes* and STEC were abundant in samples from the farm environment and were also detected in 4%, 13% and 7% of the milk filters, respectively, and in 3%, 0% and 1% of bulk tank milk samples. Four STEC isolates carried the *eae* gene, which is linked to the capacity to cause more severe human disease.

Conclusion

The results indicate a higher prevalence of *L. monocytogenes* and *Campylobacter* spp. in samples collected from loose housed herds compared to tie-stalled herds suggesting that the operating system can influence the food safety of raw milk.

Significance and Impact of the study

This study highlights that zoonotic bacteria can be present in raw milk independent of hygienic conditions at the farm and what hosing system is used. Altogether, this study provides an important knowledge base for evaluating the risk of drinking unpasteurized milk.

Keywords

Campylobacter, *Listeria*, EHEC (enterohaemorrhagic *E. coli*), Food safety, Agriculture

Introduction

Pasteurization of cow milk has been a practice in Europe since the 1880's to protect consumers from microbial pathogens (Steele 2000). Serious human diseases such as tuberculosis, brucellosis and diphtheria have dramatically decreased with the introduction of industrial methods for thermal processing of milk (Lucey 2015). As it poses a risk to public health, commercial distribution of unpasteurized milk (UPM) is legally restricted in the European Union (EU). However, since the beginning of the 21st century, consumption of UPM has grown in popularity in the Western world (Alegbeleye et al. 2018). This trend is based on the belief that UPM tastes better, has probiotic effects and is more nutritious compared to its pasteurized counterpart (Claeys et al. 2013; Crotta et al. 2016). However, there is sparse scientific evidence that support these claims. To meet consumers demands, some farmers and other actors in the agricultural community in Norway have requested relaxed rules for selling UPM (Jørgensen et al. 2005).

Cattle can be asymptomatic carriers of *Campylobacter, L. monocytogenes,* and Shiga toxin producing *E. coli* (STEC) and shed the pathogens to the farm environment via their feces. From the environment, the pathogens can spread further to the udders, milking utensils, filters and bulk storage vessels if washing and cleaning procedures are improper leading to raw milk contamination (Roberts and Wiedmann 2003; Chlebicz and Śliżewska 2018; Sapountzis et al. 2020). Several studies and reports highlight these bacteria as hazards related to consumption of UPM (De Buyser et al. 2001; Lundén et al. 2004; Langer et al. 2012; Castro et al. 2017; Artursson et al. 2018; Jaakkonen et al. 2019). *Campylobacter* is the most frequently reported cause of food poisoning in Europe (European Food Safety Authority and European Centre for

Disease Prevention and Control 2016), and isolates detected in dairy farms show genetic similarity to isolates from clinical campylobacteriosis cases (An et al. 2018). *L. monocytogenes* causes the food-borne disease listeriosis, especially in elderly, pregnant women, infants and people with weakened immune systems (Ricci et al. 2018). Some STECs can cause foodborne disease with symptoms ranging from uncomplicated diarrhea to bloody diarrhea and hemolytic uremic syndrome (HUS). Strains associated with bloody diarrhea (hemorrhagic colitis) and HUS are called enterohemorrhagic *E. coli* (EHEC). The World Health Organization (WHO) has estimated that 10% of patients with EHEC infections develop HUS with a mortality rate of 2 - 5%. The Shiga toxin (Stx) is the major virulence factor of EHEC for which, encoding genes are carried by bacteriophages (Stx phages) (Łoś et al. 2011). The adhesin; Intimin, encoded by *eae*, is another important virulence factor of EHEC necessary for enteropathogenic and enterohaemorrhagic diarrhea (Donnenberg et al. 1993; Schmidt 2010).

L. monocytogenes, *C. jejuni*- and STEC can persist in dairy farms for several months, despite good hygienic management. It has been suggested that milk contamination of STEC can be reduced by increased culling rates, improving cleaning and disinfection of barns, and by giving the livestock access to pastures (Castro et al. 2018; Jaakkonen et al. 2019). Poor-quality silage is believed to be the main reservoir for introducing *L. monocytogenes* to the dairy farm environment (Yoshida et al. 1998). Direct contamination of raw milk from cows with *Listeria* mastitis is also possible, but secondary environmental contamination of the milking instruments, where *Listeria* spp. can persist on surfaces, is probably a more relevant route of transmission to raw milk (Yoshida et al. 1998; Borucki et al. 2005). Recent studies have shown that *L. monocytogenes* is able to propagate in refrigerated milk during storage (Castro et al. 2017; Artursson et al. 2018; Castro et al. 2018). This is not the case for

Campylobacter spp. and STEC, but due to low infectious dose, propagation in food matrixes is not necessary for pathogenesis (Epps et al. 2013).

Automatic milking systems (AMS) were introduced to European dairy farms in the early 1990's, (Jacobs and Siegford 2012; Cogato et al. 2021). From year 2000, AMS have become common installations in Norwegian dairy farms and, today, more than 50% of the milk produced in Norway, originate from farms using AMS (Nørstebø et al. 2018; Hansen et al. 2019). AMS is common in farms with large herds, and loose housing allow increased contact between animals. This can lead to more problems with fecal contamination and cow cleanliness than experienced in tie stall housing systems (Hovinen et al. 2009; Hovinen and Pyörälä 2011). Several studies investigated possible connections between farm operational systems and total bacterial count (TBC) in bulk tank milk (Klungel et al. 2000; van der Vorst and Hogeveen 2000; Rasmussen et al. 2001; Rasmussen et al. 2002; de Koning et al. 2003; Van der Vorst and Ouweltjes 2003), but to our knowledge; there is limited knowledge on how transition from tie stall to loose housing influence the occurrence of zoonotic bacteria in the farm environment and in bulk tank milk. To gain more information on how farm practices and different housing systems influence the safety of raw milk, this study investigated the prevalence of *Campylobacter*, *L. monocytogenes* and STEC in raw milk and environmental samples from dairy farms representing both loose housing and tie stall housing herds. It was also determined how farm hygienic status of the herd relate to the level of zoonotic bacteria found in the raw milk and in the farm environment.

Materials and methods

To assess the food safety associated with consuming unpasteurized milk in Norway, aseptic samples of bulk tank milk (BTM), milk filters and teat milk from Norwegian dairy herds were collected and examined for presence of *L. monocytogenes*, *Campylobacter* ssp. and

Shiga toxin-producing *E. coli* (STEC). Samples were also collected from feces, feed (forage plants) and teat swabs to examine potential correlations between the presence of pathogens in the raw milk and in the farm environment. A visual evaluation of the hygienic status of the herds was performed by scoring the cleanness of the cattle at each sampling occasion. A total of 18 dairy herds from four different geographical areas in southeast of Norway were included in the study. The herds were arbitrary selected from a registry (Brønnøysundregisteret) where all Norwegian dairy-herds are registered, based on geographical selection criteria. Nine of the herds had loose housing with automatic or voluntary milking systems. Seven of the herds had tie-stall housing with CMS, where milking was performed by an operator, usually the farmer. One farm had loose housing with an integrated milking parlor, operated by the farmer and the last farm had loose housing with milking performed on a carousel operated by the farmer. In farms with loose housing systems, the number of animals ranged from 25 to 120 (mean 63) and in tie-stall farms from 19 to 33 animals (mean 25). To account for seasonal variations, each farm was sampled six times over a period of 11 months. The first sampling was performed in August and September 2019 (one farm in November), the second in November and December 2019, the third in January 2020, the fourth in February-March 2020, the fifth in May 2020 and the sixth in June 2020. Samples from bulk tank milk (BTM), milk filter, feces, feed and teats were collected at each visit, and teat milk samples were added from visit number three. After collection, all samples were kept in closed sample containers to minimize drying and exposure to air, and the samples were immediately chilled in a cooling bag containing freeze elements.

Collection of samples

BTM: A total of 200 - 400 ml of BTM was collected in sterile 50 ml tubes or in autoclaved 500 ml glass bottles at each farm visit. Fifteen of the farms had a tap connected to the cooling

tank where milk could be drained directly into the sample container. Three farms had cooling tanks with an opening on the top, where an autoclaved ladle was used to transfer milk to the sample container.

Milk filters: A disposable milk filter sock with a pore size of 100-250 µM is placed between the milking system and the bulk tank. The milk filter socks were replaced every 12 - 24 hrs and were collected at each visit. The filters were immediately cut longitudinally into three pieces (1/3 for each analysis) by a sterile scissor and directly placed in three autoclaved glass bottles, containing 200 ml of media specific for enriching either *Listeria*, *E. coli* or *Campylobacter* spp.

Feces: Fecal samples were collected from the floor of the animal houses. A total of 100 g feces was collected from five to ten different locations in each house and pooled into a sterile stomacher bag. Clean disposable plastic gloves were used during collection, and the samples were kept cool until analysis.

Feed: During each farm visit, approximately 100 g of feed (silage or silage mixture) was collected from five to ten different locations of the feed alley and pooled into a to sterile stomacher bags. Clean disposable plastic gloves were used when handling the feed samples.

Teat swabs and teat milk: Generally, 10% of the animals in each herd were sampled during each visit. However, at farms holding less than 50 animals or more than 100, the numbers of sampled animals were limited to five and ten animals, respectively. Autoclaved cotton swabs moistened in peptone water were rubbed several times across all four teats. A new swab was used for each individual animal. Swabs from different animals were then then placed into the same Falcon tube containing 15 ml peptone water and the pooled swab samples were considered to represent one herd. The teat milk samples were collected from each quarter, into sterile Falcon tubes by hand milking from the swabbed cows after disinfecting the teats

with 70% ethanol. Samples from individual cows were pooled into one sample representing the herd.

Hygiene scoring of dairy cows

A cleanliness scoring was performed on a minimum of 30% of the dairy cows in each herd. The udder, lower portions of the hind limbs and upper portions of the hind limbs and flanks, were assessed according to a four point scale, where score 0 was clean with little or no evidence of manure, 1 was clean with only slight manure splashing, 2 was dirty, distinct demarcated plaques of manure, 3 was filthy, confluent plaques of manure (Cook 2002). The score from the three zones were added and a mean score was calculated for the herd at each visit.

Isolation of *L. monocytogenes*

The samples (25 g BTM, 1/3 milk filter, 10 g feces, 10 g feed, 5 ml teat swab solution and 5 ml teat milk) were cultured for detection of *L. monocytogenes* according to the method published by the Nordic Committee on Food Analysis (NMKL) No 136, 5th ed. 2010. All samples underwent a two-step, 1:10 enrichment procedure including a primary enrichment in reduced selectivity Half Fraser broth (Oxoid, United Kingdom) at 30°C for 24 hrs, followed by enrichment in full selectivity Fraser broth (Oxoid, United Kingdom) at 37°C for 48 hrs. Cultures from the Fraser enrichments were plated on “Agar Listeria according to Agosti and Ottaviani” (ALOA) at 37°C for 24 - 48 hrs. The concentration of *L. monocytogenes* in BTM and in milk filters submerged in Half Fraser was assessed by plating 100 µl of the samples directly on ALOA. The plates were incubated at 37°C for 24 - 48 hrs before enumeration. Presumptive *L. monocytogenes* colonies from ALOA plates were confirmed after identification of beta-hemolytic, catalase positive and rhamnose positive Gram-positive rods.

Isolation of thermophilic *Campylobacter* spp.

Qualitative determination of thermotolerant *Campylobacter* was performed according to NMKL No. 119, 3. Ed., 2007, with some modifications. Samples of BTM milk (25 g), milk filters (1/3), feces (10 g), teat swab solutions (5 ml) and teat milk samples (5 ml) were transferred into Bolton broth (Oxoid, United Kingdom) for enrichment in a 1:10 ratio and then incubated at 37°C for 48 hrs in a 5% CO_2 atmosphere. The samples were further plated on selective agar mCCDA (modified charcoal cefoperazone deoxycholate agar, Oxoid, United Kingdom) and incubated at 42°C for 48 hrs in a 5% CO_2 atmosphere. For enumeration, 100 µl of milk and milk filter samples in Bolton broth were plated on mCCDA and incubated for 48 hrs at 37°C. Presumptive *Campylobacter* colonies were confirmed as *Campylobacter* spp. when they were catalase and oxidase positive and appeared as motile seagull shaped rods under phase-contrast microscopy.

Identification of STEC in samples

For enrichment of *E. coli* from either 25 ml bulk tank milk (100 samples), 1/3 of a milk filter (100 samples), or 10 g of feces (98 samples), the samples were added to 225 ml, 200 ml or 90 ml, respectively, of modified Tryptone Soya Broth (mTSB) (Oxoid, United Kingdom), supplemented with novobiocin (16 µg/ml) according to ISO/TS 13136, 1. Ed., and incubated at 37°C for 24 hrs. Each pre-culture was then divided into two parts: one part containing 1 ml that was pelleted at 13,400 rpm for 1 min for DNA isolation and polymerase chain reaction (PCR) analysis, and 1 ml for storage at -80°C until use. DNA was purified using The DNeasy® Blood and Tissue kit (Quiagen, Hilden, Germany), following the protocol for "Purification of Total DNA from Animal Tissues (Spin-Column Protocol)". Each DNA sample was examined for the presence of *stx1*, *stx2* and *eae* by PCR as described below. One µl of mTSB-enrichment cultures from samples positive for *stx*, *stx2* and *eae* were spread on

CHROMagar STEC plates (CHROMagar Microbiology, Paris, France), designed to detect multiple serotypes of STEC, including the classical O157. After incubation at 37°C for 24 hrs STECs appear on plates as mauve/pink colored colonies. Three mauve/pink colored colonies from each CHROMagar STEC plate, suspected to be a pathogenic *E. coli*, were transferred Sorbitol MacConkey Agar (SMAC) (Oxoid, UK) plates for isolation of single colonies. Non-sorbitol fermenting isolates, like STEC of serotype O157, appear with beige colonies on SMAC, while sorbitol fermenters produce pink to red colonies. Resulting single colonies isolated from SMAC were tested by PCR for detection of *stx1*, *stx2* and *eae* to identify putative potentially human pathogenic STEC isolates.

PCR

The 298 DNA samples (collected as described above) were screened for the presence of *stx1* and *stx2* by PCR by testing 1 µl of the DNA solution isolated from the mTSB sample, using Thermo Scientific DreamTaq PCR Master Mix and 0.2 µM of the corresponding primers (Additional file 1). The amplification reactions were performed in an Eppendorf Mastercycler using the following program: 3 min initial denaturation at 94°C followed by 30 cycles of denaturation at 94°C for 10 s, annealing at 52°C for 30 s and primer extension at 72°C for 60 s. The amplifications were terminated after a final elongation step of 7 min at 72°C. DNA isolated from *E. coli* O157:H7 strain EDL933 was used as a positive control. The PCR fragments were visualized by agarose gel electrophoresis.

Single colonies from the pure cultures on SMAC (transferred from CHROMagar STEC) were solved in 100 µl of H_2O, heated for 99°C for 10 min, and one µl of this sample was examined for presence of *stx1*, *stx2* and *eae* using the primes listed in Additional file 1.

Attempts to determine the serotype of the STECs by PCR were performed using the primers and conditions described by Sánchez *et al*., 2015 (Sánchez et al. 2015). The *E. coli* strains used as positive controls were of serotype O103 and O157.

Statistical analyses

A database was established in a Microsoft Excel® spreadsheet. After calculating and reviewing data in Excel, using filter functions and pivot analyses, data were transferred to STATA SE/15 (for Windows, StataCorp, College Station, TX) for further analyses. Inspection of the variables was performed in STATA using tabulations, calculations of means, medians, standard errors and 95% confidence intervals. The presence of *Campylobacter*, STEC or *L. monocytogenes* in samples were outcome variables in univariable logistic regression analyses taking account of repeated sampling by including the visits as a random variable in the models. Odds ratio (OR) are given to describe the effect of the binary variables (e.g. tie-stall versus loose housing) and coefficients are given for continuous predictors (e.g. herd size). A two-sided Fisher`s exact test was used to look for associations between the presence of pathogens in milk filter and in environmental samples (feces and fodder). Statistical significance was defined as $p<0.05$.

Results

L. monocytogenes was isolated from 79 of 556 samples (14%) and the distribution of positive samples is shown in Table 1. None of the bulk tank milk (BTM) or teat milk samples were positive for *L. monocytogenes,* but it was found in 13% of the milk filters. One farm had four *L. monocytogenes* positive milk filters and it was the only farm that had *L. monocytogenes* positive milk filters during more than one sampling occasion.

Feed samples collected in January revealed a higher occurrence of *L. monocytogenes* than those collected in August-September (coef=1.48, p=0.03) and June (coef=1.60, p=0.03). The other sample types showed no seasonal differences (Additional file 2).

The prevalence of *Campylobacter* spp. was 20% among a total of 455 tested samples (Table 2). *Campylobacter* spp. were detected in 3% of the BTM samples and 3% of the teat milk samples and in 4% of the milk filter samples. Among fecal samples, 68% were positive for *Campylobacter* spp. All farms had at least one *Campylobacter* spp. positive fecal sample during the sampling period and four farms were positive during all sampling occasions. There was no seasonal variation in the total number of samples containing *Campylobacter* spp. but the periodic sampling revealed a higher detection rate of *Campylobacter* spp. in feces during visit two/November-December and during visit five/May (Table 2, Additional file 3).

The frequency of BTM-samples, milk filter-samples and fecal-samples that were PCR positive for *stx1*and/or *stx2* and/or *eae* are given in Table 3. The highest proportion of *stx* positive samples was found in feces where 34 out of 98 samples (35%) were positive for either *stx1*, *stx2* or both. Among 100 milk filters and 100 BTM-samples, 27% and 16% respectively, were positive for either *stx1*, *stx2* or for both. In total, 12% of the milk filter samples and 10% of all samples were positive for both *stx* and *eae*.

Sixty-five out of 99 samples that were PCR positive for either *stx1*, *stx2* and/or *eae* presented typical mauve colonies on Chromagar STEC plates. Subsequent PCR analysis of single colony isolates revealed that 19 out of 298 tested samples (6%) were positive for either *stx1 or stx2*, or a combination of *stx1* and *stx2* and were, therefore, regarded as STECs (Table 4). Muliplex PCR, targeting 21 of the most clinically relevant serogroups for STEC infections in humans, revealed that the STEC isolates detected in this study did not belong to any of the seven most common serotypes O26, O45, O103, O111, O121, O145, O157, nor to the 14 remaining tested serotypes (Additional file 1).

Four out of 19 STEC isolates (21%) were positive for *eae* and were therefore considered as high-risk isolates. Three of these isolates were from the same farm and collected from two

fecal samples and one milk filter sample. The fourth isolate was detected in a fecal sample from another farm. Both of these farms were using loose housing.

No obvious seasonal variation in the prevalence of STEC was observed during the sampling period, but a tendency towards a higher proportion of *stx2* positive feces-samples was observed in the autumn compared to the spring and early summer months (Table 3). The differences between visit one (August-September) and visit five (May) (coef=-1.70, p=0.01) and six (June) (coef=-2.28, p=0.02) were statistically significant (Additional file 4). The prevalence of *eae* positive BTM samples was higher during visit one in August-September and visit six in June compared to the other samplings (Table 3) with a statistically significant difference between visit six and visit four (coef=1.92, p=0.05) (Additional file 4).

The prevalence of pathogens in milk from loose housing herds compared to tie-stall herds

L. monocytogenes was detected more frequently in fecal samples from loose housing herds compared to tie-stall herds (OR=3.19, p=.02) (Additional file 2), with an isolation prevalence of 40% and 15%, respectively (Figure 1). Farms with loose housing systems also demonstrated a higher incidence of *L. monocytogenes* in feed samples compared to farms with tie-stall systems (OR=2.75, p=.03) (Figure 1).

L. monocytogenes positive milk filters were detected in nine out of 18 farms and there was no difference in occurrence between farms with loose stall housing systems compared to those with tie stall housing systems (Figure 1). Notable, the herd which had *L. monocytogenes* positive milk filters during four sampling occasions had loose housing system. Milk filters were significantly more often positive for *L. monocytogenes* when a fecal sample (OR=6.6, p=0.01) or feed sample (OR=8.9, p=0.01) was positive for *L. monocytogenes* at the same sampling occasion. A positive association between herd size and the presence of *L.*

306 *monocytogenes* in fecal samples was observed both for loose- and tie stall systems (p=0.005
307 and p=0.043, respectively).

308 There was a clear difference in the occurrence of *Campylobacter* spp. in fecal samples from
309 farms with loose housing systems compared to those with tie-stall housing (OR=3.65,
310 p<.001) (Figure 1, Additional file 3). Similarly, there was a higher occurrence of
311 *Campylobacter* spp. in teat swabs from farms with loose housing compared to those with tie
312 stall housing (OR=9.70, p=0.03). There was, however, no significant difference in the
313 prevalence of *Campylobacter* spp. in milk filters (p=0.52) or teat milk samples (p=0.76)
314 between farms having loose housing versus tie-stall systems. Neither farms with loose
315 housing nor those with tie stall housing showed an association between herd size and the
316 occurrence of *Campylobacter* species in feces samples. There was, however, an association
317 between the isolation rate of *Campylobacter* spp. in teat swabs (coef=0.03, p<0.001) and herd
318 size regardless of housing system.

319 *Campylobacter* spp. was detected in milk filters from four out of 18 herds; one of these had
320 tie stall housing and three had loose housing. A two-sided Fisher exact test did not show an
321 association between positive fecal samples and positive milk filter samples (OR=1.02,
322 p=1.00), but there were too few positive milk filters to look for a correlation with
323 environmental samples.

324 STECs were isolated from fecal samples collected from four loose housing herds and from
325 two tie-stall herds. However, four out of 11 STEC positive fecal samples (36%) came from
326 one specific farm where the animals were tie stalled. STECs were also isolated from seven
327 milk filters distributed over 5 out of 18 herds; one of these herds had tie-stall housing while
328 four had loose housing. Notably, one STEC positive bulk tank milk sample was collected

from a loose housed herd. The four *stx* and *eae* positive samples were collected from loose housing herds.

Association between dairy cow hygiene score and detection of pathogenic bacteria in dairy farm samples

During sampling, the hygienic status of each cattle herd was scored (0-9) and the mean score from six sampling occasions are shown in Figure 2. We observed an association (coef=0.83, p=0.03) between dairy cow hygiene score and detection of *Campylobacter* spp. in teat milk samples (Figure 2, Additional file 3). There was also a trend towards a positive association between dairy cow hygiene score and detection of *Campylobacter* spp. in milk filters (coef=0.56, p=0.06). No obvious association between hygiene score and detection of *L. monocytogenes* or *Campylobacter* species in BTM, feces, feed or teat swab was observed (Figure 2). Furthermore, no coherence was seen between dairy cow hygiene score and detection of STEC from BTM, milk filters or feces. However, a p-value of 0.06 (coef=0.37), may indicate a correlation between hygiene score and the number of *eae* positive feces samples (Additional file 4). Interestingly, the farm with the lowest dairy cow hygiene score had the third lowest *L. monocytogenes* detection rate (6% positive), and STEC was not detected in any of the samples from this farm. *Campylobacter* spp. were detected in 24% of the samples from this herd, the fifth highest detection rate of all farms included in the study.

Discussion

To explore the risk associated with consumption of UPM in Norway, the occurrence of *L. monocytogenes*, *Campylobacter* ssp. and STEC in Norwegian dairy herds and in raw milk was examined. Eighteen different farms, located in a radius of 100 km around Oslo, were

352 included in the study. The included farms are regarded representative for this region but may
353 not represent the total dairy cattle population in Norway due to geographical and climatic
354 differences. *Salmonella* spp. were not included in this study as the Norwegian cattle
355 population is only sporadically infected with pathogens from this group (Norwegian
356 Veterinary Institute 2019).

357 Consumption of milk and dairy products have been associated with approximately half of all
358 foodborne *L. monocytogenes* outbreaks in Europe, which makes it a serious public health
359 concern (De Buyser et al. 2001; Lundén et al. 2004). In this study, *L. monocytogenes* was
360 isolated from 13% of the milk filters but it was not found in any of the BTM samples. A
361 similar occurrence was reported from a Swedish study from 2018 which detected *L.*
362 *monocytogenes* in 7% of the milk filter samples but not in the BTM samples (Artursson et al.
363 2018). Studies from other European countries have found *L. monocytogenes* in UPM samples
364 with a prevalence of 1- 4% (Beckers et al. 1987; Waak et al. 2002). A higher prevalence of *L.*
365 *monocytogenes* was reported from a Finnish study, which found *L. monocytogenes* in 29% of
366 milk filter samples and 13% of BTM samples from three dairy farms (Castro et al. 2018). An
367 American study from 2018, found *L. monocytogenes* in 2.5% of milk filter samples and in
368 1.1% BTM samples (Sonnier et al. 2018), which is similar to what was reported from
369 European studies (Beckers et al. 1987; Waak et al. 2002; Artursson et al. 2018). The
370 detection of *L. monocytogenes* in the milk filter samples in all these studies strongly indicate
371 that this bacterium is often present in milking systems. The low prevalence of *L.*
372 *monocytogenes* detected in BTM in the present study is most likely due to a dilution effect
373 and small testing volumes and do not exclude the presence of *L. monocytogenes* in BTM. The
374 absence of *L. monocytogenes* in teat milk is compatible with *Listeria* being an environmental
375 challenge rather than being a component of the normal udder flora, which supports the
376 importance of good milking hygiene.

In this study, we detected *Campylobacter* spp. in 4% of the milk filter samples, in 3% of the BTM samples, and in 68% of the fecal samples. For comparison, a study from Finland reported the prevalence of *C. jejuni* in milk filter samples to be less than 1%; it was not found in BTM samples but was present in 53% of fecal samples (Jaakkonen et al. 2019). In Swedish study, *C. jejuni* was detected in 7% of milk filters but not in BTM samples (Artursson et al. 2018). The farms included in the Finnish study (Jaakkonen et al. 2019) tested positive for *C. jejuni* and STEC O157:H7 prior to the study took place and had already introduced strict hygienic measures to get rid of the problem, which might have led to underestimation of the pathogen-prevalence relative to more normal settings. In the Finnish and the Swedish study, the identity of *C. jejuni* was confirmed by MALDI biotyping and pulsed-field gel electrophoresis (PFGE), respectively, but in the present study, it was only identified to the level of "thermophilic *Campylobacter* spp." which may also include other *Campylobacter* spp. than *C. jejuni*.

Campylobacteriosis has for many years been the most commonly reported gastrointestinal disease in the EU (European Food Safety Authority and European Centre for Disease Prevention and Control 2018), and outbreaks associated with consumption of unpasteurized milk have frequently been reported (Lehner et al. 2000; Harrington et al. 2002; Schildt et al. 2006; Heuvelink et al. 2009; Kenyon et al. 2020). In 2017, 66 Danish school children got campylobacteriosis after visiting a farm where they had raw milk served directly from the barn (Statens Serum Institut 2018). A similar outbreak occurred in Sweden in 2014, where eleven people, seven of them young children, fell ill after consumption of UPM after visiting a dairy farm (Lahti et al. 2017). Altogether, based on the current and previous studies we propose a continued awareness regarding the risk of campylobacteriosis related to consumption of unpasteurized milk.

One of the most important health-threats associated with consumption of UPM is STEC. Cattle are a natural reservoir of STEC, and approximately 75% of EHEC outbreaks are linked to consumption of contaminated beef and milk products (Sperandio and Nguyen 2012). This study showed an STEC occurrence of 7%, 1% and 11% in milk filter, BTM, and feces samples, respectively. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks announce that 8.1% of European cattle tested positive for STEC in 2017 (European Food Safety Authority and European Centre for Disease Prevention and Control 2018), which is similar to what was found in the present study. In a study from Finland, Jaakkonen and coworkers (Jaakkonen et al. 2019) isolated 2% and 0% of STEC O157 and 1% and 0% of non-O157 STECs from milk filters and BTM, respectively, which is a slightly lower occurrence than observed in the present study. We have, however, used a different approach to identify STEC than was used in the Finnish study, as we omitted the immunomagnetic separation step, which selects for certain serotypes. The inclusion of all *stx* positive isolates, regardless of serotype, could at least partly explain the higher STEC prevalence obtained in this study. The first described *E. coli* causing enterohemorrhagic disease and HUS was of serotype O157:H7 (Riley et al. 1983) but non-O157 STEC infections have increasingly been reported over the last decade (Brooks et al. 2005; Hughes et al. 2006; Gould et al. 2013). Since new EHEC variants are continuously emerging, each STEC strain should be considered as a potential EHEC (Bielaszewska et al. 2011; Rasko et al. 2011). Notably, even the presence of low levels of EHEC in UPM can pose a serious risk, particularly for individuals belonging to the high-risk group) as it has a low infectious dose of only 10-100 bacteria (Sperandio and Nguyen 2012).

The primer-panel used for geno-serotyping is described by Sánchez et al. (Sánchez et al. 2015), and is designed to identify 21 clinically relevant serogroups of STEC. It was, however, not possible to identify the serotypes of the STECs isolated in this study by using this primer

panel, which indicate that they belong to other serotypes than those that are identified by this method. Notably, as many as 187 *E. coli* serogroups have been described based on nucleotide sequences of the O-antigen gene cluster (DebRoy et al. 2016) and, out of these, 158 are known to carry the Shiga toxin gene(s) (Ludwig et al. 2020).

Previous studies have reported *stx* gene prevalence's of 7-15% for BTM samples and 40-50% for milk filter samples (Van Kessel et al. 2011; Jaakkonen et al. 2019). In the present study, 20% of all BTM samples and 34% of all milk filter samples were PCR positive for *stx*. Notably, as *stx* genes are carried by bacteriophages, free phage particles will also result in a positive detection when PCR screening samples. Therefore, it is important to keep in mind that food samples that are PCR positive for *stx*, do not necessarily represent a direct risk to human health but should rather be interpreted as a sign of increased risk of occurrence of STEC. Intimin, encoded by *eae*, is necessary for intimate attachment of enteropathogenic *E. coli* (EPEC) to epithelial cells (Donnenberg et al. 1993). Approximately ¼ of the milk filter samples in this study were positive for *eae*, indicating a high likelihood for the presence of Intimin positive *E. coli* strains (also called enteropathogenic *E. coli*) in the raw milk. This study also identified an *eae* positive STEC isolate from a milk filter sample, indicating a high possibility of presence of EHEC in raw milk.

To explore the differences in pathogen occurrence in farms with different operating systems both tie-stall and loose stall herds were included in the study. Statistical analysis revealed that the occurrence of *Campylobacter* spp. in feces and teat swabs and *L. monocytogenes* in feces and feed was higher in loose housed herds compared to tie-stall herds. Confounding factors, like herd size, may at least partly explain the difference in occurrence as loose housed herds often are of larger size compared to tie-stalled, which confers more animal-to-animal interactions and increased fecal contamination of the environment.

Several studies have shown a correlation between the hygiene score of the udder and hind limb and somatic cell count (Valde et al. 1997; Reneau et al. 2005; Cook and Reinemann 2006; Dohmen et al. 2010). One would expect that animals with a poor hygiene score would carry more pathogenic bacteria and represent an increased risk for contaminating the milk during milking. Cleanliness varies with season and with the surrounding environment. Dairy cattle at farms with loose housing may perform better on a hygiene scoring in the summer season when the animals have access to outdoor grazing and the animal density is reduced in the traffic alleys, feeding- and bedding- areas inside. The hygiene score is also likely to depend on the state of the pastures. Rainy summers often causes pastures to be muddy, which will reduce the cleanliness score considerably. Cattle in tie-stall housings, with good management practices, often appear cleaner in the indoor season when they are more separated from other animals and it is easier to control the environmental hygiene when the animals are tied up.

The feed samples showed a seasonal variation in the presence of *L. monocytogenes*, with higher levels in the winter months November/December, January, and February/March (33%, 56% and 33%, respectively) compared to August/September, May and June (22%, 21% and 20%, respectively). Notably, only January compared to September and June were statistically significant ($P = 0.03$). Similar seasonal variations were also reported by a Finnish study which detected higher levels of *L. monocytogenes* in milk filters during the indoor season. A study from New York state (USA), reported a higher prevalence of *L. monocytogenes* during the winter season in samples collected from cattle and small-ruminant farms (Nightingale et al. 2005; Castro et al. 2018). However, there are also reports which did not find any seasonal variations in the prevalence of *L. monocytogenes* at dairy farms (Gaya et al. 1998; Hassan et al. 2001; Mohammed et al. 2010) and some studies found higher *L. monocytogenes* levels during the summer season (Hutchison et al. 2005; Dalzini et al. 2016). Differences in study

design and local climate conditions could be factors that account for the discrepancy regarding seasonal variations in *L. monocytogenes* levels reported from different studies.

In conclusion, the present study reveals a wide distribution of *L. monocytogenes*, *Campylobacter* spp. and STEC in environmental samples collected at Norwegian dairy farms, independent of housing system. The presence of bacteria with low infectious doses in milking systems combined with a human population of increasing age and with more people suffering from underlaying risk factors for severe disease, reinforce the importance of strict regulations regarding commercial sales of UPM. The evolvement of agricultural technologies will most probably continue to present new food safety challenges in the future and the need for continuous adaptation of hygiene measures and pathogen control strategies must be highlighted.

Acknowledgements

We thank Nofima and Helga Ness for the use of the Biosafety lab for the isolation of STEC and Anette Wold for helpful instructions. The authors thank Marte Monshaugen and Kristin O`Sullivan for technical support. We thank Eystein Skjerve for expertise in designing the database, and Elínborg Steinunn Pálsdóttir and Henriette Sofie Ross Pedersen for help during sampling. Finally, we thank all the dairy farmers for their generosity and for providing the sample material to the study.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be interpreted as a potential conflict of interest.

References

497 Alegbeleye, O.O., Guimarães, J.T., Cruz, A.G. and Sant'Ana, A.S. (2018) Hazards of a
498 'healthy' trend? An appraisal of the risks of raw milk consumption and the potential
499 of novel treatment technologies to serve as alternatives to pasteurization. *Trends Food*
500 *Sci Technol* 82, 148-166.
501 An, J.U., Ho, H., Kim, J., Kim, W.H., Kim, J., Lee, S., Mun, S.H., Guk, J.H., Hong, S. and
502 Cho, S. (2018) Dairy Cattle, a Potential Reservoir of Human Campylobacteriosis:
503 Epidemiological and Molecular Characterization of *Campylobacter jejuni* From Cattle
504 Farms. *Front Microbiol* 9, 3136.
505 Artursson, K., Schelin, J., Thisted Lambertz, S., Hansson, I. and Olsson Engvall, E. (2018)
506 Foodborne pathogens in unpasteurized milk in Sweden. *Int J Food Microbiol*, 284,
507 120-127.
508 Beckers, H.J., Soentoro, P.S.S. and Delgou-van Asch, E.H.M. (1987) The occurrence of
509 *Listeria monocytogenes* in soft cheeses and raw milk and its resistance to heat. *Int J*
510 *Food Microbiol* 4, 249-256.
511 Bielaszewska, M., Mellmann, A., Zhang, W., Köck, R., Fruth, A., Bauwens, A., Peters, G.
512 and Karch, H. (2011) Characterisation of the *Escherichia coli* strain associated with
513 an outbreak of haemolytic uraemic syndrome in Germany, 2011: a microbiological
514 study. *Lancet Infect Dis* 11, 671-676.
515 Borucki, M.K., Gay, C.C., Reynolds, J., McElwain, K.L., Kim, S.H., Call, D.R. and
516 Knowles, D.P. (2005) Genetic diversity of *Listeria monocytogenes* strains from a
517 high-prevalence dairy farm. *Appl Environ* 71, 5893-5899.
518 Brooks, J.T., Sowers, E.G., Wells, J.G., Greene, K.D., Griffin, P.M., Hoekstra, R.M. and
519 Strockbine, N.A. (2005) Non-O157 Shiga toxin–producing *Escherichia coli* infections
520 in the United States, 1983–2002. *J Infect Dis* 192, 1422-1429.

Castro, H., Ruusunen, M. and Lindström, M. (2017) Occurrence and growth of *Listeria monocytogenes* in packaged raw milk. *Int J Food Microbiol* 261, 1-10.

Castro, H., Jaakkonen, A., Hakkinen, M., Korkeala, H. and Lindström, M. (2018) Occurrence, Persistence, and Contamination Routes of *Listeria monocytogenes* Genotypes on Three Finnish Dairy Cattle Farms: a Longitudinal Study. *Appl Environ Microbiol* 84, e02000-17.

Chlebicz, A. and Śliżewska, K. (2018) Campylobacteriosis, Salmonellosis, Yersiniosis, and Listeriosis as Zoonotic Foodborne Diseases: A Review. *Int J Environ Res Public Health* 15, 863.

Claeys, W.L., Cardoen, S., Daube, G., De Block, J., Dewettinck, K., Dierick, K., De Zutter, L., Huyghebaert, A., Imberechts, H. and Thiange, P. (2013) Raw or heated cow milk consumption: Review of risks and benefits. *J Food control* 31, 251-262.

Cogato, A., Brščić, M., Guo, H., Marinello, F. and Pezzuolo, A. (2021) Challenges and Tendencies of Automatic Milking Systems (AMS): A 20-Years Systematic Review of Literature and Patents. *Animals* (Basel), 11, 356.

European Food Safety Authority and European Centre for Disease Prevention and Control (2016) The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2015. *EFSA J*, 14, e04634.

European Food Safety Authority and European Centre for Disease Prevention and Control (2018) The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2017. *EFSA J*, 16, e05500-n/a.

Cook, N.B. and Reinemann, D. (2006) *A Tool Box for Assessing Cow, Udder and Teat Hygiene.* Annual meeting of the National Mastitis Council, Madison, Wisconsin, USA.

Cook, N. B. (2002) *The influence of Barn Design on Dairy Cow Hygiene, Lameness and Udder Health*. American Association of Bovine Practitioners thirty-fifth annual conference, Madison, Wisconsin, USA.

Crotta, M., Paterlini, F., Rizzi, R. and Guitian, J. (2016) Consumers' behavior in quantitative microbial risk assessment for pathogens in raw milk: Incorporation of the likelihood of consumption as a function of storage time and temperature. *J Dairy Sci*, 99, 1029-1038.

Dalzini, E., Bernini, V., Bertasi, B., Daminelli, P., Losio, M.N. and Varisco, G. (2016) Survey of prevalence and seasonal variability of *Listeria monocytogenes* in raw cow milk from Northern Italy. *Food Control*, 60: 466-470.

De Buyser, M.L., Dufour, B., Maire, M. and Lafarge, V. (2001) Implication of milk and milk products in food-borne diseases in France and in different industrialised countries. *Int J Food Microbiol* 67, 1-17.

de Koning, K., Slaghuis, B. and van der Vorst, Y. (2003) Robotic milking and milk quality. Effects on bacterial counts - somatic cell counts - freezing point and free fatty acids. *Ital J Anim Sci* 2, 291-299.

DebRoy, C., Fratamico, P.M., Yan, X., Baranzoni, G., Liu, Y., Needleman, D.S., Tebbs, R., O'Connell, C.D., Allred, A. and Swimley, M. (2016) Comparison of O-antigen gene clusters of all O-serogroups of Escherichia coli and proposal for adopting a new nomenclature for O-typing. *PLoS One* 11, e0147434.

Dohmen, W., Neijenhuis, F. and Hogeveen, H. (2010) Relationship between udder health and hygiene on farms with an automatic milking system. *J Dairy Sci*, 93, 4019-4033.

Donnenberg, M.S., Yu, J. and Kaper, J.B. (1993) A second chromosomal gene necessary for intimate attachment of enteropathogenic *Escherichia coli* to epithelial cells. *J Bacteriol* 175, 4670-4680.

570 Epps, S., Harvey, R., Hume, M., Phillips, T., Anderson, R. and Nisbet, D. (2013) Foodborne
571 Campylobacter: Infections, Metabolism, Pathogenesis and Reservoirs. *Int J Environ*
572 *Res Public Health* 10, 6292-6304.
573 Gaya, P., Sanchez, J., Medina, M. and Nuñez, M. (1998) Incidence of *Listeria*
574 *monocytogenes* and other Listeria species in raw milk produced in Spain. *Food*
575 *microbiol* 15, 551-555.
576 Gould, L.H., Mody, R.K., Ong, K.L., Clogher, P., Cronquist, A.B., Garman, K.N., Lathrop,
577 S., Medus, C., Spina, N.L. and Webb, T.H. (2013) Increased recognition of non-O157
578 Shiga toxin–producing *Escherichia coli* infections in the United States during 2000–
579 2010: epidemiologic features and comparison with *E. coli* O157 infections.
580 *Foodborne Pathog Dis* 10, 453-460.
581 Hansen, B.G., Herje, H.O. and Höva, J. (2019) Profitability on dairy farms with automatic
582 milking systems compared to farms with conventional milking systems. *Int Food*
583 *Agribusiness Manag*, 22, 215-228.
584 Harrington, P., Archer, J., Davis, J.P., Croft, D.R. and Varma, J. K. (2002) Outbreak of
585 *Campylobacter jejuni* infections associated with drinking unpasteurized milk procured
586 through a cow-leasing program--Wisconsin, 2001. *Morb Mortal Wkly Rep* No. 0149-
587 2195, pp.548-549. Centers for Disease Control Prevention.
588 Hassan, L., Mohammed, H.O. and McDonough, P.L. (2001) Farm-management and milking
589 practices associated with the presence of *Listeria monocytogenes* in New York state
590 dairy herds. *Prev Vet Med* 51, 63-73.
591 Heuvelink, A.E., van Heerwaarden, C., Zwartkruis-Nahuis, A., Tilburg, J.J.H.C., Bos, M.H.,
592 Heilmann, F.G.C., Hofhuis, A., Hoekstra, T. and de Boer, E. (2009) Two outbreaks of
593 campylobacteriosis associated with the consumption of raw cows' milk. *Int J Food*
594 *Microbiol* 134, 70-74.

595 Hovinen, M., Rasmussen, M.D. and Pyörälä, S. (2009) Udder health of cows changing from
596 tie stalls or free stalls with conventional milking to free stalls with either conventional
597 or automatic milking. *J Dairy Sci* 92, 3696-3703.
598 Hovinen, M. and Pyörälä, S. (2011) Invited review: Udder health of dairy cows in automatic
599 milking. *J Dairy Sci* 94, 547-562.
600 Hughes, J.M., Wilson, M.E., Johnson, K.E., Thorpe, C.M. and Sears, C.L. (2006) The
601 emerging clinical importance of non-O157 Shiga toxin—producing *Escherichia coli*.
602 *Clin Infect Dis* 43, 1587-1595.
603 Hutchison, M.L., Walters, L.D., Avery, S.M., Munro, F. and Moore, A. (2005) Analyses of
604 livestock production, waste storage, and pathogen levels and prevalences in farm
605 manures. *Appl Environ Microbiol* 71, 1231-1236.
606 Statens Serum Institut (2018) Campylobacter infections, 2016-2017. *Annual reports on*
607 *disease incidence*. Denmark.
608 Jacobs, J.A. and Siegford, J.M. (2012) Invited review: The impact of automatic milking
609 systems on dairy cow management, behavior, health, and welfare. *J Dairy Sci* 95,
610 2227-2247.
611 Jørgensen, H.J., Mørk, T. and Rørvik, L.M. (2005) The Occurrence of *Staphylococcus aureus*
612 on a Farm with Small-Scale Production of Raw Milk Cheese. *J Dairy Sci* 88, 3810-
613 3817.
614 Jaakkonen, A., Castro, H., Hallanvuo, S., Ranta, J., Rossi, M., Isidro, J., Lindström, M. and
615 Hakkinen, M. (2019) Longitudinal Study of Shiga Toxin-Producing *Escherichia coli*
616 and *Campylobacter jejuni* on Finnish Dairy Farms and in Raw Milk. *Appl Environ*
617 *Microbiol* 85, e02910-18.

618 Kenyon, J., Inns, T., Aird, H., Swift, C., Astbury, J., Forester, E. and Decraene, V. (2020)
619 Campylobacter outbreak associated with raw drinking milk, North West England,
620 2016. *Epidemiol Infect* 148, e13.
621 Klungel, G., Slaghuis, B. and Hogeveen, H. (2000) The effect of the introduction of
622 automatic milking systems on milk quality. *J Dairy Sci* 83, 1998-2003.
623 Lahti, E., Rehn, M., Ockborn, G., Hansson, I., Ågren, J., Engvall, E.O. and Jernberg, C.
624 (2017) Outbreak of campylobacteriosis following a dairy farm visit: confirmation by
625 genotyping. *Foodborne Pathog Dis* 14, 326-332.
626 Langer, A.J., Ayers, T., Grass, J., Lynch, M., Angulo, F.J. and Mahon, B.E. (2012)
627 Nonpasteurized dairy products, disease outbreaks, and state laws-United States, 1993-
628 2006. *Emerg Infect Dis* 18, 385-391.
629 Lehner, A., Schneck, C., Feierl, G., Pless, P., Deutz, A., Brandl, E. and Wagner, M. (2000)
630 Epidemiologic application of pulsed-field gel electrophoresis to an outbreak of
631 *Campylobacter jejuni* in an Austrian youth centre. *Epidemiol Infect* 125, 13-16.
632 Łoś, J. M., Łoś, M. and Węgrzyn, G. (2011) Bacteriophages carrying Shiga toxin genes:
633 genomic variations, detection and potential treatment of pathogenic bacteria. *Future*
634 *microbiol* 6, 909-924.
635 Lucey, J.A. (2015) Raw Milk Consumption: Risks and Benefits. *Nutr Today* 50, 189-193.
636 Ludwig, J.B., Shi, X., Shridhar, P.B., Roberts, E.L., DebRoy, C., Phebus, R.K., Bai, J. and
637 Nagaraja, T.G. (2020) Multiplex PCR assays for the detection of one hundred and
638 thirty seven serogroups of Shiga toxin-producing *Escherichia coli* associated with
639 cattle. *Front Cell Infect Microbiol* 10, 378.
640 Lundén, J., Tolvanen, R. and Korkeala, H. (2004) Human listeriosis outbreaks linked to dairy
641 products in Europe. *J Dairy Sci* 87, e6-e12.

642 Mohammed, H.O., Atwill, E., Dunbar, L., Ward, T., McDonough, P., Gonzalez, R. and
643 Stipetic, K. (2010) The risk of Listeria monocytogenes infection in beef cattle
644 operations. *J Appl Microbiol* 108, 349-356.
645 Nightingale, K.K., Fortes, E.D., Ho, A.J., Schukken, Y.H., Grohn, Y.T. and Wiedmann, M.
646 (2005) Evaluation of farm management practices as risk factors for clinical listeriosis
647 and fecal shedding of *Listeria monocytogenes* in ruminants. *J Am Vet Med Assoc* 227,
648 1808-1814.
649 Norwegian Veterinary Institute (2019) The surveillance programmes for Salmonella in live
650 animals, eggs and meat in Norway 2019, annual report. p.3.
651 Nørstebø, H., Rachah, A., Dalen, G., Rønningen, O., Whist, A. C. and Reksen, O. (2018)
652 Milk-flow data collected routinely in an automatic milking system: an alternative to
653 milking-time testing in the management of teat-end condition? *Acta Vet Scand* 60, 2-
654 9.
655 Rasko, D.A., Webster, D.R., Sahl, J.W., Bashir, A., Boisen, N., Scheutz, F., Paxinos, E.E.,
656 Sebra, R., Chin, C.S. and Iliopoulos, D. (2011) Origins of the *E. coli* strain causing an
657 outbreak of hemolytic–uremic syndrome in Germany. *N Engl J Med* 365, 709-717.
658 Rasmussen, M.D., Blom, J.Y., Nielsen, L.A.H. and Justesen, P. (2001) Udder health of cows
659 milked automatically. *Livest Prod Sci* 72, 147-156.
660 Rasmussen, M.D., Bjerring, M., Justesen, P. and Jepsen, L. (2002) Milk quality on Danish
661 farms with automatic milking systems. *J Dairy Sci* 85, 2869-2878.
662 Reneau, J.K., Seykora, A.J., Heins, B.J., Endres, M.I., Farnsworth, R.J. and Bey, R.F. (2005)
663 Association between hygiene scores and somatic cell scores in dairy cattle. *J Am Vet*
664 *Med Assoc* 227, 1297-1301.
665 Ricci, A., Allende, A., Bolton, D., Chemaly, M., Davies, R., Fernández Escámez, P.S.,
666 Girones, R., Herman, L., Koutsoumanis, K., Nørrung, B., et al. (2018) *Listeria*

667 *monocytogenes* contamination of ready-to-eat foods and the risk for human health in
668 the EU. *EFSA J* 16, e05134-n/a.
669 Riley, L.W., Remis, R.S., Helgerson, S.D., McGee, H.B., Wells, J.G., Davis, B.R., Hebert,
670 R.J., Olcott, E.S., Johnson, L.M. and Hargrett, N.T. (1983) Hemorrhagic colitis
671 associated with a rare *Escherichia coli* serotype. *N Engl J Med* 308, 681-685.
672 Roberts, A.J. and Wiedmann, M. (2003) Pathogen, host and environmental factors
673 contributing to the pathogenesis of listeriosis. *Cell Mol Life Sci* 60, 904-918.
674 Sánchez, S., Llorente, M.T., Echeita, M.A. and Herrera-León, S. (2015) Development of
675 three multiplex PCR assays targeting the 21 most clinically relevant serogroups
676 associated with Shiga toxin-producing *E. coli* infection in humans. *PLoS One* 10,
677 e0117660-e0117660.
678 Sapountzis, P., Segura, A., Desvaux, M. and Forano, E. (2020) An Overview of the Elusive
679 Passenger in the Gastrointestinal Tract of Cattle: The Shiga Toxin Producing
680 *Escherichia coli*. *Microorganisms* (Basel) 8, 877.
681 Schildt, M., Savolainen, S. and Hänninen, M.L. (2006) Long-lasting *Campylobacter jejuni*
682 contamination of milk associated with gastrointestinal illness in a farming family.
683 *Epidemiol Infect* 134, 401-405.
684 Schmidt, M.A. (2010) LEEways: tales of EPEC, ATEC and EHEC. *Cell Microbiol* 12, 1544-
685 1552.
686 Sonnier, J.L., Karns, J.S., Lombard, J.E., Kopral, C.A., Haley, B.J., Kim, S.W. and Van
687 Kessel, J.A.S. (2018) Prevalence of *Salmonella enterica*, *Listeria monocytogenes*, and
688 pathogenic *Escherichia coli* in bulk tank milk and milk filters from US dairy
689 operations in the National Animal Health Monitoring System Dairy 2014 study. *J*
690 *Dairy Sci* 101, 1943-1956.

691 Sperandio, V. and Nguyen, Y. (2012) Enterohemorrhagic *E. coli* (EHEC) pathogenesis. *Front*
692 *Cell Infect Microbiol* 2, 90.
693 Steele, J.H. (2000) History, trends, and extent of pasteurization. *J Am Vet Med Assoc* 217,
694 175-178.
695 Valde, J.P., Hird, D.W., Thurmond, M.C. and Oesteraas, O. (1997). Comparison of ketosis,
696 clinical mastitis, somatic cell count, and reproductive performance between free stall
697 and tie stall barns in Norwegian dairy herds with automatic feeding. *Acta Vet Scand*
698 38, 181-192.
699 van der Vorst, Y. and Hogeveen, H. (2000) *Automatic milking systems and milk quality in the*
700 *Netherlands*. Robotic milking: Proceedings of the International Symposium held in
701 Lelystad, The Netherlands, 17-19 August, 2000. Wageningen Pers. pp.73-82.
702 Van der Vorst, Y. and Ouweltjes, W. (2003) Milk quality and automatic milking; a risk
703 inventory. Lelystad, The Netherlands. Praktijkonderzoek Veehouderij.
704 Van Kessel, J.A.S., Karns, J.S., Lombard, J.E. and Kopral, C.A. (2011) Prevalence of
705 *Salmonella enterica*, *Listeria monocytogenes*, and *Escherichia coli* Virulence Factors
706 in Bulk Tank Milk and In-Line Filters from U.S. Dairies. *J Food Prot* 74, 759-768.
707 Waak, E., Tham, W. and Danielsson-Tham, M.L. (2002) Prevalence and Fingerprinting of
708 *Listeria monocytogenes* Strains Isolated from Raw Whole Milk in Farm Bulk Tanks
709 and in Dairy Plant Receiving Tanks. *Appl Environ Microbiol* 68, 3366-3370.
710 Yoshida, T., Kato, Y., Sato, M. and Hirai, K. (1998) Sources and routes of contamination of
711 raw milk with *Listeria monocytogenes* and its control. *J Vet Med Sci* 60, 1165-1168.

712
713

Tables

Table 1 Occurrence of *Listeria monocytogenes* in dairy farm samples

Sampling	BTM	MF	Feces	Silage	Teat swab	Teat milk	Total
1	0/18 (0)	0/17 (0)	5/16 (31)	4/18 (22)	0/17 (0)		9/86 (10)
2	0/18 (0)	4/16 (25)	6/18 (33)	6/18 (33)	1/18 (6)		17/88 (19)
3	0/18 (0)	2/17 (12)	4/18 (22)	10/18 (56)	1/18 (6)	0/18 (0)	17/107 (16)
4	0/18 (0)	1/13 (8)	7/18 (39)	6/18 (33)	1/18 (6)	0/18 (0)	15/103 (15)
5	0/14 (0)	2/14 (14)	2/14 (14)	3/14 (21)	2/14 (14)	0/14 (0)	9/84 (11)
6	0/16 (0)	3/16 (19)	6/15 (40)	3/15 (20)	0/13 (0)	0/13 (0)	12/88 (14)
Total	0/102 (0)	12/93 (13)	30/99 (30)	32/101 (32)	5/98 (5)	0/63 (0)	79/556 (14)

Proportion (%) of bulk tank milk- (BTM), milk filter- (MF), feces-, silage-, teat swab- and teat milk-samples positive for *Listeria monocytogenes*. The samples were collected at six different time points between August 2019 and July 2020.

Table 2 Occurrence of *Campylobacter* spp. in dairy farm samples

Sampling	BTM	MF	Feces	Teat swab	Teat milk	Total
1	2/18 (11)	2/17 (12)	9/16 (56)	1/17 (6)		14/68 (21)
2	1/18 (6)	1/16 (6)	15/18 (83)	2/18 (11)		19/70 (27)
3	0/18 (0)	0/17 (0)	11/18 (61)	3/18 (17)	1/18 (6)	15/89 (17)
4	0/18 (0)	1/13 (8)	12/18 (67)	3/18 (17)	1/18 (6)	17/85 (20)
5	0/14 (0)	0/14 (0)	12/14 (86)	3/14 (21)	0/14 (0)	15/70 (21)
6	0/16 (0)	0/16 (0)	8/15 (53)	1/13 (8)	0/13 (0)	9/73 (12)
Total	3/102 (3)	4/93 (4)	67/99 (68)	13/98 (13)	2/63 (3)	89/455 (20)

Number (%) of bulk tank milk- (BTM), milk filter- (MF), feces, teat swab- and teat milk-samples positive for *Campylobacter* spp.. The samples were collected at six different time points between August 2019 and July 2020.

725 **Table 3** Occurrence of *stx1*, *stx2* and *eae* in dairy farm samples

Sample	Visit	*stx1*	*stx2*	*eae*
Feces	1	5/15 (33)	9/15 (60)	3/15 (20)
	2	6/18 (33)	8/18 (44)	4/18 (22)
	3	3/18 (17)	5/18 (28)	3/18 (17)
	4	1/18 (6)	5/18 (28)	2/18 (11)
	5	2/14 (14)	3/14 (21)	1/14 (7)
	6	2/15 (13)	2/15 (13)	1/15 (7)
	Total	19/98 (19)	32/98 (33)	14/98 (14)
Milk filter	1	2/18 (11)	7/18 (39)	5/18 (28)
	2	2/18 (11)	4/18 (22)	5/18 (28)
	3	1/18 (6)	3/18 (17)	4/18 (22)
	4	2/16 (13)	2/16 (13)	3/16 (19)
	5	1/14 (7)	3/14 (21)	0/14 (0)
	6	1/16 (6)	6/16 (38)	7/16 (44)
	Total	9/100 (9)	25/100 (25)	24/100 (24)
Bulk tank milk	1	0/18 (0)	2/18 (11)	5/18 (28)
	2	0/18 (0)	1/18 (6)	2/18 (11)
	3	0/18 (0)	0/18 (0)	2/18 (11)
	4	1/18 (6)	2/18 (11)	1/18 (6)
	5	0/14 (0)	1/14 (7)	1/14 (7)
	6	9/14 (64)	4/14 (29)	4/14 (29)
	Total	10/100 (10)	10/100 (10)	15/100 (15)
All samples	Total	38/298 (13)	67/298 (22)	53/298 (18)

726 Number (%) of bulk tank milk-samples, milk filter-samples and fecal-samples positive for *stx1*, *stx2*

727 and *eae*.

728 **Table 4** Occurrence of Shiga toxin-producing *Escherichia coli* in BTM, milk filters and feces

Sampling	BTM	MF	Feces	Total all samples
1	0/18	1/18 (6)	1/15 (7)	2/51 (4)
2	0/18	1/18 (6)	3*/18 (17)	4/54 (7)
3	0/18	1/18 (6)	2*/18 (11)	3/54 (6)

4	0/18	0/16	1/18 (6)	1/52 (2)
5	0/14	1/14 (7)	2/14 (14)	3/42 (7)
6	1/14 (7)	3*/16 (19)	2*/15 (13)	6/45 (13)
Total	1/100 (1)	7/100 (7)	11/98 (11)	19/298 (6)

729 Number (%) of Shiga toxin producing *E. coli* (STEC) isolates from bulk tank milk (BTM) samples,
730 milk filter (MF) samples and fecal samples positive for *stx1*, *stx2* and *eae*. **E. coli* isolates positive
731 for both *stx* and *eae* were isolated from three fecal samples (sampling 2, 3 and 6) and from one milk
732 filter (sampling 6).

733

Figure 1

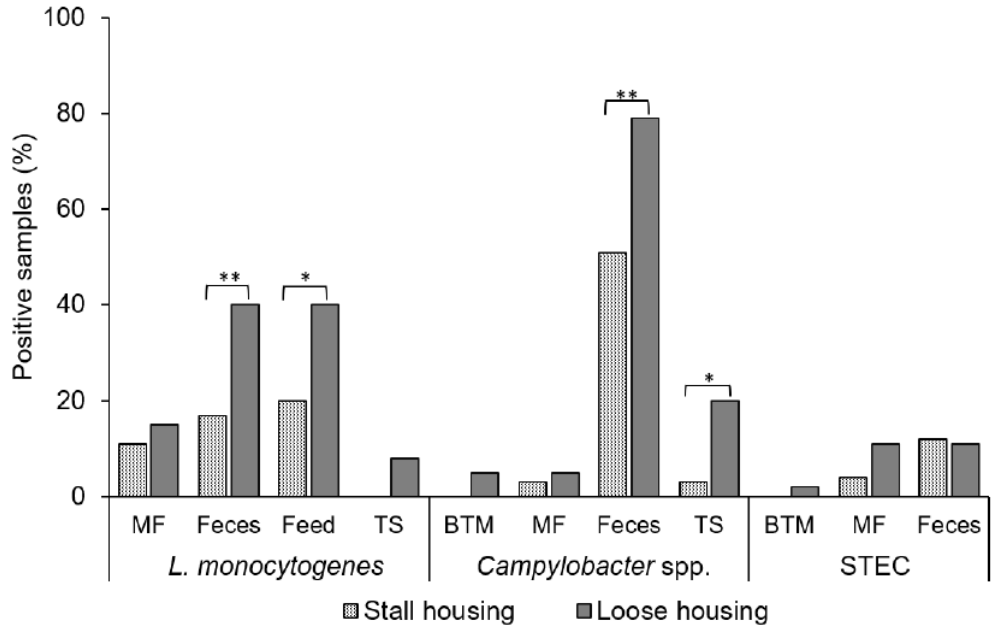
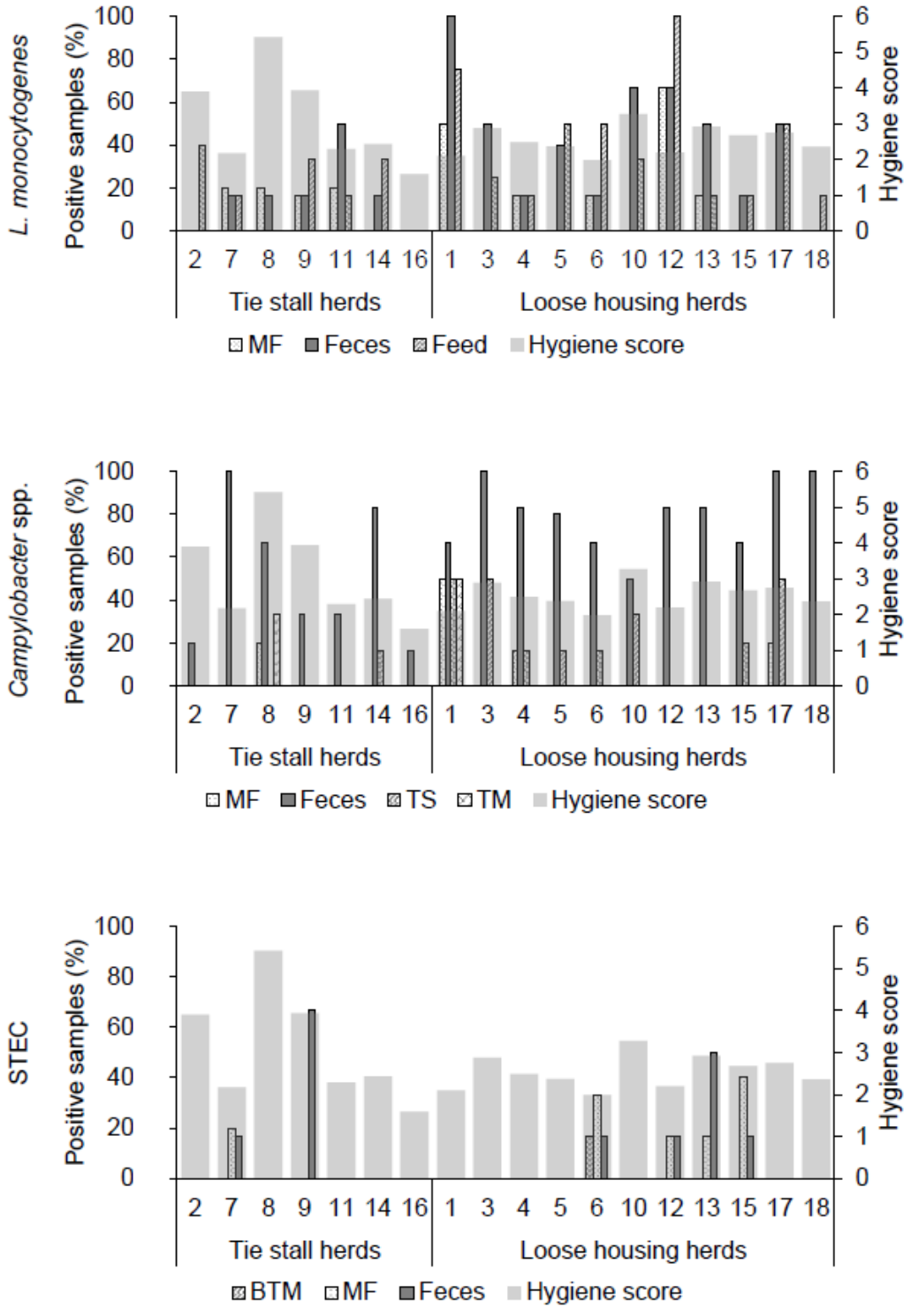


Figure 1. Pathogen occurrence according to housing system

Number of (%) samples positive for *Listeria monocytogenes*, *Campylobacter* spp. and Shiga toxin-producing *E. coli* in loose housing versus tie-stall housing (*;p>.05, **;p>.02). MF = milk filter, BTM = bulk tank milk, TS = teat swab.

754 **Figure 2**



755

756 Figure 2. Pathogen occurrence versus dairy cattle hygiene score

757 % samples positive for *Listeria monocytogenes*, *Campylobacter* spp. and Shiga toxin-
758 producing *E. coli* in each herd together with average dairy cattle hygiene score. MF = milk
759 filter, BTM = bulk tank milk, TS = teat swab, TM = teat milk.

Supporting information

Additional file 1

Primers used in the study

Additional file 2

Statistics on *L. monocytogenes* occurrence

Additional file 3

Statistics on *Campylobacter* spp. occurrence

Additional file 4

Statistics on *stx* and *eae* occurrence

Additional file 5

Statistics on STEC occurrence

Table S1 Primers used in the study

Primer name	Primer sequence (5´-3´)	Reference
Stx2F	GCGTTTTGACCATCTTCGT	[39]
Stx2R	ACAGGAGCAGTTTCAGACAG	[39]
Stx1F	GATAGTGGCTCAGGGGATAAT	This study
Stx1R	GCCGAAAACGTAAAGCTTCAG	This study
EaeF	GTGGCGAATACTGGCGAGACT	[40]
EaeR	CTTGTGCGCTTTGGCTTC	This study
O5	CTTATCCGATTAATGGCTTC	[38]
O5	TAGTCGATTTGCTTTTATGG	[38]
O91F	TTTTCTGGAATGCTTGATGA	[38]
O91R	ATAATTTTACGCCGTGTTTG	[38]
O26F	ACTCTTGCTTCGCCTGTT	[38]
O26R	CAGCGATACTTTGAACCTTAT	[38]
O103F	TATCCTTCATAGCCTGTTGTT	[38]
O103R	TTATAATAGTAATAAGCCAGACACC	[38]
O145F	TTGAGCACTTATCACAAGAGATT	[38]
O145R	GATTGAATAGCTGAAGTCATACTAAC	[38]
O121	GTAGCGAAAGGTTAGACTGG	[38]
O121	ATGGGAAAGCTGATACTGC	[38]
O111F	GTTGCGAGGAATAATTCTTCA	[38]
O111R	CCATAGATATTGCATAAAGGC	[38]
O55	ATCGCAATTGCAATAAACTC	[38]
O55	CCCAACTCTAGTAGATAAAAGCC	[38]
O128F	TTTCGATCGTCTTGTTCAGG	[38]
O128R	CAATGGGCAATTAACACAGAG	[38]
O113	TAACGGGATTAGAAGTGGAT	[38]
O113	ATATAAGGCAGAAATGAGAGG	[38]
O146	ATCAGTTCATGGGTTGTATTC	[38]
O146	AGGAACATGGATGAAAGAAG	[38]
O45	GACTTTCGTTGCGTTGTG	[38]
O45	CTGCAAGTGTAGCGAAAAC	[38]
O177	TCGGTGTTTGAAGGGGAAG	[38]

O177	GTCCATGCATATGCCGTTC	[38]
O157F	CTCAATTTATAAAAAAGACGCTC	[38]
O157R	TCCAAATATTAACGACTTCACTAC	[38]
O15	GCGTTGCCTACTTACTTATTATC	[38]
O15	ATGCAAGTCCAGCCAAAC	[38]
O104F	CGGTGTATTAAGAAGTGTTGTC	[38]
O104R	ATACTCCCCATAGAAACGC	[38]
O118F	TGGAGAACAGATAGCAAGAGG	[38]
O118R	TATCCGACAAACACGAACC	[38]
O123	GAAAGAACAGAATCAGACTATGC	[38]
O123	TGTGCTAGCGCTAAAGGAC	[38]
O165	AACTGTTTATCCGAAGTGGTAG	[38]
O165	CACGCTTTAACGCATACAG	[38]
O172	ATTGGGTAGCCTCAGTAAAG	[38]
O172	CAGTCCAAACAGTGACAGTATC	[38]

Table S2 Statistics on *L. monocytogenes* occurrence

Predictor variable	**Random effect**	**Outcome variable**	**Odds ratio**	**Coefficient**	**Std. Err.**	**z**	**P-value**	**95% Lo**	**95% Hi**
Loose vs tie stall housing		Feces	3.19		1.58	2.35	0.02	1.21	8.41
		Silage	2.75		1.30	2.13	0.03	1.09	6.96
		Milk filter	1.45		0.94	0.57	0.57	0.40	5.20
Herd size	Herd number	Feces		0.03	0.01	4.12	<0.01	0.02	0.05
		Silage		0.01	0.01	1.84	0.07	<-0.01	0.03
		Milk filter		0.01	0.01	0.68	0.50	-0.01	0.03
		Teat swab		0.02	0.01	1.83	0.07	0.00	0.04
Dairy cow cleanliness score 0-9	Visit	Feces		-0.17	0.18	-0.98	0.33	-0.52	0.18
		Milk filter		-0.20	0.28	-0.74	0.46	-0.75	0.34
		Teat swab		-0.11	0.37	-0.30	0.76	-0.83	0.61
Visit 3 vs 1	Herd number	Silage		1.48	0.68	2.18	0.03	0.15	2.80
Visit 3 vs 6				1.6	0.75	2.15	0.03	0.14	3.08
Feces* pos or neg		Milk filter	6.6				<0.01	1.50	32.49
Feed* pos or neg			8.85				<0.01	1.91	54.22

*Fisher exact test

Table S3 Statistics on *Campylobacter* spp. occurrence

Predictor variable	Random effect	Outcome variable	Odds ratio	Coefficient	Std. Err.	z	P-value	95% Lo	95% Hi
Loose vs stall housing		Feces	3.65		1.64	2.88	<0.01	1.51	8.82
		Teat milk	0.65		0.93	-0.30	0.76	0.04	10.87
		Milk filter	2.13		2.51	0.65	0.52	0.21	21.34
		Teat swab	9.70		10.32	2.14	0.03	1.21	78.00
Herd size	Herd number	Feces		0.01	0.01	0.74	0.46	-0.02	0.04
		Teat milk		0.02	0.02	0.97	0.33	-0.02	0.07
		Milk filter		0.02	0.01	1.40	0.16	-0.01	0.05
		Bulk tank milk		0.03	0.01	2.24	0.03	<0.01	0.05
		Teat swab		0.03	0.01	4.59	<0.01	0.02	0.04
Dairy cow cleanliness score	Visit	Feces		-0.09	0.17	-0.56	0.58	-0.43	0.24
		Teat milk		0.83	0.38	2.21	0.03	0.10	1.57
		Milk filter		0.56	0.30	1.86	0.06	-0.03	1.15
		Bulk tank milk		-0.02	0.46	-0.05	0.96	-0.93	0.89
		Teat swab		-0.14	0.24	-0.59	0.56	-0.61	0.33
Season	Visit 2 vs 1	Feces		1.36	0.57	2.37	0.018	0.23	2.48
	Visit 2 vs 6			1.48	0.76	1.95	0.051	-0.01	2.96
	Visit 5 vs 1			1.54	0.74	2.08	0.037	0.09	2.99
	Visit 5 vs 6			1.66	0.72	2.29	0.022	0.24	3.08

Table S4 Statistics on *stx* and *eae* occurrence

Predictor variable	**Random effect**	**Outcome variable**		**Odds ratio**	**Coefficient**	**Std. Err.**	**z**	**P-value**	**95% Lo**	**95% Hi**
Loose vs stall housing		Feces	*stx*	1.04		0.45	0.10	0.92	0.45	2.42
			eae	1.97		1.24	1.07	0.28	0.57	6.78
		Milk filter	*stx*	1.85		0.90	1.28	0.20	0.72	4.78
			eae	3.24		1.79	2.13	0.03	1.10	9.59
		Bulk tank milk	*stx*	1.57		0.92	0.78	0.44	0.50	4.93
			eae	5.26		4.15	2.10	0.04	1.12	24.73
Herd size		Feces	*stx*	0.99		0.01	-1.07	0.28	0.98	1.01
			eae	1.00		0.01	0.41	0.68	0.99	1.02
		Milk filter	*stx*	0.995		0.01	-0.56	0.58	0.98	1.01
			eae	1.01		0.01	1.48	0.14	1.00	1.03
		Bulk tank milk	*stx*	0.99		0.01	-1.17	0.24	0.96	1.01
			eae	1.00		0.01	0.08	0.94	0.98	1.02
Dairy cow cleanliness score	Visit	Feces	*stx*		0.12	0.16	0.76	0.45	-0.19	0.43
			eae		0.37	0.20	1.87	0.06	-0.02	0.76
		Milk filter	*stx*		-0.19	0.19	-1.02	0.31	-0.56	0.18
			eae		-0.01	0.19	-0.07	0.95	-0.38	0.35
		Bulk tank milk	*stx*		-0.41	0.35	-1.19	0.24	-0.01	0.27
			eae		-0.28	0.25	-1.09	0.27	-0.77	0.22

Visit 5 vs 1		Feces	*stx2*		-1.70	0.61	-2.77	0.006	-2.91	-0.50
Visit 6 vs 1		Feces	*stx2*		-2.28	0.97	-2.35	0.019	-4.17	-0.38
Visit 4 vs 1		Bulk tank milk	*stx1*		-3.42	1.10	-3.12	0.002	-5.57	-1.27
Visit 6 vs 4		Bulk tank milk	*eae*		1.92	0.96	1.99	0.047	0.03	3.81

Table S5 Statistics on STEC occurrence

Predictor variable	Random effect	Outcome variable	Odds ratio	Coefficient	Std. Err.	z	P-value	95% Lo	95% Hi
Loose vs stall housing		Milk filter	4.33		4.77	1.33	0.18	0.50	37.45
		Feces	0.85		0.55	-0.26	0.80	0.24	2.99
Herd size	Herd number	Milk filter		-0.02	0.02	-1.29	0.20	-0.05	0.01
		Feces		-0.01	0.02	-0.82	0.41	-0.05	0.02
Dairy cow cleanliness score 0-9	Visit	Milk filter		-0.42	0.37	-1.15	0.25	-1.15	0.30
		Feces		0.21	0.21	1.00	0.32	-0.20	0.63
Visit 5 vs 1	Herd number	Milk filter		0.27	0.14	1.94	0.053	<-0.01	0.54