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

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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 housing 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

67 Disease Prevention and Control 2016), and isolates detected in dairy farms show genetic
68 similarity to isolates from clinical campylobacteriosis cases (An et al. 2018). *L.*
69 *monocytogenes* causes the food-borne disease listeriosis, especially in elderly, pregnant
70 women, infants and people with weakened immune systems (Ricci et al. 2018). Some STECs
71 can cause foodborne disease with symptoms ranging from uncomplicated diarrhea to bloody
72 diarrhea and hemolytic uremic syndrome (HUS). Strains associated with bloody diarrhea
73 (hemorrhagic colitis) and HUS are called enterohemorrhagic *E. coli* (EHEC). The World
74 Health Organization (WHO) has estimated that 10% of patients with EHEC infections
75 develop HUS with a mortality rate of 2 - 5%. The Shiga toxin (Stx) is the major virulence
76 factor of EHEC for which, encoding genes are carried by bacteriophages (Stx phages) (Łoś et
77 al. 2011). The adhesin; Intimin, encoded by *eae*, is another important virulence factor of
78 EHEC necessary for enteropathogenic and enterohaemorrhagic diarrhea (Donnenberg et al.
79 1993; Schmidt 2010).

80 *L. monocytogenes*, *C. jejuni*- and STEC can persist in dairy farms for several months, despite
81 good hygienic management. It has been suggested that milk contamination of STEC can be
82 reduced by increased culling rates, improving cleaning and disinfection of barns, and by
83 giving the livestock access to pastures (Castro et al. 2018; Jaakkonen et al. 2019). Poor-
84 quality silage is believed to be the main reservoir for introducing *L. monocytogenes* to the
85 dairy farm environment (Yoshida et al. 1998). Direct contamination of raw milk from cows
86 with *Listeria* mastitis is also possible, but secondary environmental contamination of the
87 milking instruments, where *Listeria* spp. can persist on surfaces, is probably a more relevant
88 route of transmission to raw milk (Yoshida et al. 1998; Borucki et al. 2005). Recent studies
89 have shown that *L. monocytogenes* is able to propagate in refrigerated milk during storage
90 (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* spp. 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

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

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

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

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

156 Teat swabs and teat milk: Generally, 10% of the animals in each herd were sampled during
157 each visit. However, at farms holding less than 50 animals or more than 100, the numbers of
158 sampled animals were limited to five and ten animals, respectively. Autoclaved cotton swabs
159 moistened in peptone water were rubbed several times across all four teats. A new swab was
160 used for each individual animal. Swabs from different animals were then then placed into the
161 same Falcon tube containing 15 ml peptone water and the pooled swab samples were
162 considered to represent one herd. The teat milk samples were collected from each quarter,
163 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% CO₂ 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% CO₂ 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 H₂O, 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).

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

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

272 Sixty-five out of 99 samples that were PCR positive for either *stx1*, *stx2* and/or *eae* presented
273 typical mauve colonies on Chromagar STEC plates. Subsequent PCR analysis of single
274 colony isolates revealed that 19 out of 298 tested samples (6%) were positive for either *stx1*
275 or *stx2*, or a combination of *stx1* and *stx2* and were, therefore, regarded as STECs (Table 4).

276 Multiplex PCR, targeting 21 of the most clinically relevant serogroups for STEC infections in
277 humans, revealed that the STEC isolates detected in this study did not belong to any of the
278 seven most common serotypes O26, O45, O103, O111, O121, O145, O157, nor to the 14
279 remaining tested serotypes (Additional file 1).

280 Four out of 19 STEC isolates (21%) were positive for *eae* and were therefore considered as
281 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

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

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

377 In this study, we detected *Campylobacter* spp. in 4% of the milk filter samples, in 3% of the
378 BTM samples, and in 68% of the fecal samples. For comparison, a study from Finland
379 reported the prevalence of *C. jejuni* in milk filter samples to be less than 1%; it was not found
380 in BTM samples but was present in 53% of fecal samples (Jaakkonen et al. 2019). In Swedish
381 study, *C. jejuni* was detected in 7% of milk filters but not in BTM samples (Artursson et al.
382 2018). The farms included in the Finnish study (Jaakkonen et al. 2019) tested positive for *C.*
383 *jejuni* and STEC O157:H7 prior to the study took place and had already introduced strict
384 hygienic measures to get rid of the problem, which might have led to underestimation of the
385 pathogen-prevalence relative to more normal settings. In the Finnish and the Swedish study,
386 the identity of *C. jejuni* was confirmed by MALDI biotyping and pulsed-field gel
387 electrophoresis (PFGE), respectively, but in the present study, it was only identified to the
388 level of “thermophilic *Campylobacter* spp.” which may also include other *Campylobacter*
389 spp. than *C. jejuni*.

390 Campylobacteriosis has for many years been the most commonly reported gastrointestinal
391 disease in the EU (European Food Safety Authority and European Centre for Disease
392 Prevention and Control 2018), and outbreaks associated with consumption of unpasteurized
393 milk have frequently been reported (Lehner et al. 2000; Harrington et al. 2002; Schildt et al.
394 2006; Heuvelink et al. 2009; Kenyon et al. 2020). In 2017, 66 Danish school children got
395 campylobacteriosis after visiting a farm where they had raw milk served directly from the
396 barn (Statens Serum Institut 2018). A similar outbreak occurred in Sweden in 2014, where
397 eleven people, seven of them young children, fell ill after consumption of UPM after visiting
398 a dairy farm (Lahti et al. 2017). Altogether, based on the current and previous studies we
399 propose a continued awareness regarding the risk of campylobacteriosis related to
400 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 underlying 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.

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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.

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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	<i>stx1</i>	<i>stx2</i>	<i>eae</i>
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.

760 **Supporting information**

761 Additional file 1

762 Primers used in the study

763 Additional file 2

764 Statistics on *L. monocytogenes* occurrence

765 Additional file 3

766 Statistics on *Campylobacter* spp. occurrence

767 Additional file 4

768 Statistics on *stx* and *eae* occurrence

769 Additional file 5

770 Statistics on STEC occurrence

771

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	CCCAACTCTAGTAGATAAAAAGCC	[38]
O128F	TTTCGATCGTCTTGTTTCAGG	[38]
O128R	CAATGGGCAATTAACACAGAG	[38]
O113	TAACGGGATTAGAAGTGGAT	[38]
O113	ATATAAGGCAGAAATGAGAGG	[38]
O146	ATCAGTTCATGGGTGTATTC	[38]
O146	AGGAACATGGATGAAAGAAG	[38]
O45	GACTTTCGTTGCGTTGTG	[38]
O45	CTGCAAGTGTAGCGAAAAC	[38]
O177	TCGGTGTTTGAAGGGGAAG	[38]

O177	GTCCATGCATATGCCGTTT	[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	<i>stx2</i>		-1.70	0.61	-2.77	0.006	-2.91	-0.50
Visit 6 vs 1		Feces	<i>stx2</i>		-2.28	0.97	-2.35	0.019	-4.17	-0.38
Visit 4 vs 1		Bulk tank milk	<i>stx1</i>		-3.42	1.10	-3.12	0.002	-5.57	-1.27
Visit 6 vs 4		Bulk tank milk	<i>eae</i>		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