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ORIGINAL ARTICLE

Occurrence of *Campylobacter* spp., *Salmonella* spp. and shiga toxin-producing *Escherichia coli* in inline milk filters from Swedish dairy farms

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Abstract

This study investigated the occurrence of shiga toxin-producing *Escherichia coli* (STEC), thermotolerant *Campylobacter* spp. and *Salmonella* spp. in Swedish dairy milk. A total of 302 inline milk filters were analyzed. Salmonella was not isolated from any filters. Polymerase chain reaction screening detected thermotolerant Campylobacter in 30.5% of the milk filters analyzed and it was isolated from 12.6% of filters. The *stx* genes (stx_1, stx_2, or both) were screened from 71% of the filters and STEC was isolated from 14% of these. Of the STEC isolates, 21 contained the stx_1 gene, 19 the stx_2 gene, and five a combination of both stx_1 and stx_2 genes. Whole genome sequence typing on 34 of the 45 STEC showed that they belonged to 21 different serotypes, of which STEC O145:H28 was the most common (2%). STEC O157:H7 was only found from one (0.3%) of the filters. A combination of stx_2 and *eae* genes was found from 0.7% of the total number of inline milk filters analyzed, while stx_{2a} was found in 24% of the whole genome-sequenced isolates. There was a significant positive correlations between number of animals per farm and presence of pathogens on milk filters.

1 | INTRODUCTION

Fresh dairy milk is a commonly consumed food in Sweden, with per capita consumption of almost 90 L per year (SCB, 2018a). Milk is a nutrient-rich product containing high-quality protein and the majority of the vitamins and minerals needed for a complete diet (Pereira, 2014). However, milk is a complex food that can contain a wide variety of microorganisms, including lactic acid bacteria, spoilage organisms, and potential pathogens (EFSA, 2015; Quigley et al., 2013). Pathogens can contaminate milk either directly from the udder of infected animals or indirectly from the dairy farm environment during and after milking (Oliver, Jayarao, & Almeida, 2005; van Kessel, Karns, Gorski, McCluskey, & Perdue, 2004). Due to the near neutral pH, high water activity, and high nutrient composition, pathogens can easily multiply in milk stored at inappropriate temperatures (EFSA, 2015). There have been many studies on the occurrence of pathogens in unpasteurized dairy milk, in which *Campylobacter* spp., shiga toxin-producing *Escherichia coli* (STEC), and *Salmonella* spp. are commonly reported (FSAI, 2015; Claeys et al., 2013; D'Amico, Groves, & Donnelly, 2008; Giacometti et al., 2013; Hill, Smythe, Lindsay, & Shepherd, 2012; Jackson et al., 2012; Mohammadi, Abiri, Rezaei, & Salmanzadeh-Ahrabi, 2013; Ruusunen et al., 2013; Schoder, Maichin, Lema, & Laffa, 2013; Zastempowska, Grajewski, & Twarużek, 2016).

Campylobacter spp. is the most common bacterial cause of gastroenteritis in Sweden, with approximately 100 cases per 100,000 inhabitants and year between 2015 and 2017 (EFSA & ECDC, 2017; SVA, 2016). Human campylobacteriosis is mainly attributed to consumption of undercooked meat, especially poultry, but consumption of unpasteurized milk is a known mode of transmission (Batz, Hoffmann, & Morris, 2012; Costard, Espejo, Groenendaal, & Zagmutt, 2017; Pires, Vigre, Makela, & Hald, 2010). In the United States, it is estimated that consumption of unpasteurized milk and cheese increases the risk of campylobacteriosis

by 7,600-fold (Costard et al., 2017). In Sweden, an outbreak with eight confirmed cases of *Campylobacter jejuni* occurred in 2014. It was attributed to consumption of unpasteurized milk by a preschool class (2–7 years old) during a farm visit (Lahti et al., 2017). The incidence of human illness due to STEC in Sweden is lower than that due to *Campylobacter* spp., with only approximately 4–6 cases per 100,000 inhabitants and year between 2015 and 2017 (EFSA & ECDC, 2017; SVA, 2016). However, STEC infection can cause serious disease in humans and is associated with major costs to both the individual and society. Children are at particular risk of developing severe symptoms, such as hemolytic uremic syndrome (HUS) and other sequelae (Germinario et al., 2016). Because of the potential severity of the disease, it is essential to reduce the number of cases of STEC infection. STEC infection is mainly attributed to consumption of raw/undercooked meat, cured meat/cold cuts, or minced meat from cattle or small ruminants (Mughini-Gras et al., 2018). However, consumption of unpasteurized cow's milk has also been reported as a transmission route for STEC infection (Costard et al., 2017; Germinario et al., 2016; Jaakkonen et al., 2017).

Swedish legislation states that fresh milk sold at retail must be pasteurized. However, there is growing consumer interest in purchasing unpasteurized milk and it is legally permissible for producers in Sweden to sell up to 70 L of unpasteurized milk directly to consumers on farm. However, both STEC and *Campylobacter* spp. have been shown to cause illness after ingestion of only a low number of cells, and therefore even small amounts of contaminated milk can lead to consumer infection (Paton & Paton, 1998; Robinson, 1981). Consumption of unpasteurized milk has been correlated with both sporadic cases and outbreaks of foodborne illness. Between 2007 and 2012, a total of 24 and 81 outbreaks caused by unpasteurized milk were reported in the EU and US, respectively (EFSA, 2015; Mungai, Behravesh, & Gould, 2015). *Campylobacter* spp. was reported to be the causative agent in the majority of these outbreaks, followed by STEC and *Salmonella* Typhimurium.

The aim of the present study was to investigate the occurrence of STEC, thermotolerant *Campylobacter* spp., and *Salmonella* spp. in Swedish dairy milk. This information is needed in order to provide relevant support to risk managers and as input to the ongoing debate on whether a more relaxed approach to selling unpasteurized milk should be adopted in Sweden.

2 | MATERIAL AND METHODS

2.1 | Selection of farms and sampling

The study was performed in three regions southern Sweden: Skåne, Västra Götaland, and Öland. These regions were selected due to their high percentage of dairy farms and previously reported occurrence of STEC O157:H7 clade 8 and *Salmonella* (Ågren, Lewerin, Wahlström, Emanuelson, & Frössling, 2016; Boqvist, Aspan, & Eriksson, 2009; Eriksson, Aspan, Gunnarsson, & Vågsholm, 2005). Herds were randomly selected from among dairy producers in each region, in order to obtain samples of milk filters from herds of different sizes and from farms using different management practices. Participation in the study was voluntary for the selected dairy farmers and farms that were under investigation for *Salmonella* were excluded from the study. Information on the milking system (automatic, manual, untethered, tethered) and the number of dairy cows was obtained from each farm.

Inline milk filters were collected by the farmers directly after the morning milking, and placed in zipper bags containing 50 ml of Cary-Blair transport medium (National Veterinary Institute [SVA], Uppsala, Sweden). The bags were then stored in a refrigerator until later in the same day, when they were sent by post to the Swedish Food Agency in freezer boxes containing cooling brackets. Criteria for inclusion in the study were that the transport time did not exceed 24 hr and that the temperature of the samples was below 8°C on arrival at the laboratory.

2.2 | Preparation of the inline milk filters before examination

On arrival at the laboratory, the inline milk filters were cut into three approximately equal sections, using sterilized scissors. These sections were to be used for detection of *Salmonella*, thermotolerant *Campylobacter*, and STEC, respectively, as described below. If more than one filter was delivered from a specific farm, only one was analyzed.

2.3 | Detection of *Salmonella*

One filter section was mixed with 150 ml buffered peptone water (Oxoid, Waltham, MA), homogenized, and pre-enriched at 37 ± 1°C for 16–20 hr. Genomic DNA was extracted from the pre-enrichment broth and detection of *Salmonella* spp. was performed with real-time polymerase chain reaction (PCR; Bio-Rad CFX96™ Real-Time System; Bio-Rad Laboratories, Hercules, CA) using the iQ-check Salmonella II kit and Standard I protocol (Bio-Rad Laboratories) according to the manufacturer's instructions. In cases of positive PCR detection of *Salmonella* spp., an attempt was made to isolate the strains involved. In brief, 0.1 ml of the pre-enrichment was transferred to tubes containing Rappapport-Vassiliadis soy peptone broth (Oxoid) and incubated at 42 ± 0.2°C for 21–27 hr. Enriched broth was then streaked out on selective xylose lysine deoxycholate (Oxoid) agar and Brilliance™ Salmonella (Oxoid) agar plates, which were incubated at 37 ± 1°C for 21–27 hr according to NMKL 71 (1999).

2.4 | Detection of thermotolerant *Campylobacter*

For *Campylobacter* detection, one filter section was mixed into 150 ml Bolton broth (Oxoid) and the broth was incubated for 44–52 hr at 41.5 ± 1°C in a jar with microaerobic atmosphere (5% O_2, 10% CO_2, and 85% N_2). Genomic DNA was purified with Chelex-based resin from 1 ml of the enriched broth, according to the manufacturer's instructions for Instagene™ Matrix (Bio-Rad Laboratories, Hercules, CA). Detection of thermotolerant *Campylobacter* was performed by real-time PCR (Bio-Rad and Roche, Basel, Switzerland), based on amplification of a 287-bp sequence of the 16S rRNA gene of *C. jejuni*, *C. coli*, and *C. lari*. The PCR was performed as described by Josefsen, Jacobsen, and Hoorfar (2004), with the modification that TaqMan

Universal Mastermix (Applied Biosystems, Foster City, CA), primer concentration of 0.5 μM and probe concentration of 0.1 μM was used. In cases of positive PCR detection of *Campylobacter*, an attempt was made to isolate the strains according to NMKL 119 (2007). In brief, 10 μl of enriched broth were streaked on modified charcoal cephoperazone desoxycolate agar plates (Oxoid), which were incubated at 41.5 ± 1°C in a jar with modified atmosphere (5% O_2, 10% CO_2, and 85% N_2) at 44–52 hr. Presence of *Campylobacter* spp. was assessed based on motility, cell morphology, and the Bactident® oxidase test (Merck, Kenilworth, NJ). Identification of *C. jejuni* and *C. coli* was performed by real-time PCR with designed primers and probes according to Toplak, Kovač, Piskernik, Smole Možina, and Jeršek (2012), with the modifications that PerfeCTa MultiPlex qPCR SuperMix (Quantabio, Beverly, MA) was used and the primer and probe concentration was set to 0.8 and 0.2 μM, respectively, for detection of *C. jejuni* and to 0.3 and 0.2 μM, respectively, for detection of *C. coli*. Identification was also performed by mass spectrometry-time of flight (Maldi-Tof; Bruker Corporation, Billerica, MA) at SVA according to their instructions (Holmberg, Rosendal, Engvall, Ohlson, & Lindberg, 2015). The characteristic patterns of *Campylobacter* spp. proteins were matched using the FDA-cleared Reference Library from Bruker and the SVA library.

2.5 | Detection of shiga toxin-producing *Escherichia coli*

The filter section for STEC analysis was mixed with 150 ml modified Tryptone Soy Broth (Oxoid) containing 0.225 g bile salt no. 3 (Oxoid) and 12 mg/L acriflavin (Sigma-Aldrich, Saint Louis, MO). After incubation at 37 ± 1°C for 18–24 hr, genomic DNA was extracted from 200 μl enriched broth using a BioRobot EZ1 (Qiagen, Hilden, Germany) and EZ1 DNA Tissue kit (Qiagen) according the manufacturer's instructions. The extracted genomic DNA was eluted in 100 μl elution buffer. Detection of the genes stx_1 and stx_2 was performed using the real-time PCR (Bio-Rad and Roche) method described in ISO/TS 13136:2012 and Kagkli et al. (2011). Upon detection of one or both genes, real-time PCR for the genes of the five serogroups O157, O26, O103, O111, and O145 was performed (ISO/TS 13136, 2012; Perelle, Dilasser, Grout, & Fach, 2004, 2005), with the modification that the annealing temperature was set to 60°C for all targets.

In the event of detection of shiga toxin genes, the enrichment broth was frozen with 20% glycerol at −70°C. When enough positive samples were collected, an attempt was made to isolate through immunoblotting according to Atalla and Johnson (2000). In brief, the capture membrane (82 mm nitrocellulose membranes, pore size 0.2 μm; VWR International) was precoated with rabbit anti-Stx antibodies (2 μg/ml) and blocked with wash buffer containing 1% gelatin. The enrichment broth was thawed at 50°C and left at room temperature for 1 hr (Ternent et al., 2003), and then diluted 10-fold in peptone water (Oxoid) containing 1% NaCl (Merck). The capture membrane was positioned on Tryptic Soy Agar plates (Oxoid) containing 25 ng/ml Mitomycin C (Sigma-Aldrich). Above the capture membrane, a second uncoated membrane (82 mm cellulose acetate, pre size 0.45 μm; Satorius Group, Göttingen, Germany) was positioned. A volume of 100 μl from selected dilutions was spread onto the membranes and incubated at 37°C for 18–24 hr. The membranes were marked for later reorientation. The capture membrane was then removed and the upper membrane was replaced on the tryptic soy agar plates and stored at 4°C for later use. A mixture of monoclonal antibodies against Stx_1, $Stx_{2a/c}$, Stx_{2e}, and Stx_{2d}-variants (2 μg/ml) was used for the capture membrane as the secondary antibody, followed by alkaline phosphatase-labeled rabbit anti-mouse IgG (0.1 μg/ml; Jackson Immunoresearch, Mississauga, Ontario, Canada), and BCIP/NBT (5-bromo-4-chloro-3-indoyl-phosphate/nitroblue tetrazolium; Seracare, Milford, MA) was used for detection. Suspected colonies from the immunoblotting were streaked on tryptone bile x-glucoronide agar (Oxoid) and also confirmed as *E. coli* by testing for production of indole, confirmed with real-time PCR for the *stx* genes as described above.

Confirmed isolates were sent to the Public Health Agency of Sweden for characterization with whole genome sequencing. For this, Ion Torrent 400 base-pair chemistry was used, together with Library Builder™ and Ion Torrent platform (Thermo Fischer). In a first step, the information from sequencing was used to determine species and then characterized with respect to serotype, *stx*-subtype, and the virulence genes *eae* and *aggR*.

2.6 | Statistical analyses

Chi^2 tests were used in all comparisons to determine significant differences in relation to occurrence of specific bacteria and time of sampling (spring and autumn), region, and herd size. Results were considered significant at $p < 0.05$.

3 | RESULTS

3.1 | Farms and samples included in the survey

In total, inline filters from 302 randomly selected dairy farms were analyzed, of which 139 were sampled from April–June 2015 and 163 sampled from August–October 2015. The information obtained for these samples is summarized in Table 1.

3.2 | *Salmonella*

With PCR screening, *Salmonella* spp. was detected in 0.7% (2/232) of the filters. However, it was not possible to isolate *Salmonella* from the PCR-positive samples.

3.3 | Thermotolerant *Campylobacter*

Through PCR screening, thermotolerant *Campylobacter* was detected in 30.5% (92/302) of the milk filters and successfully isolated from 12.6% (38 /302) of the milk filters (Table 2). The majority of the isolates (90%) were *C. jejuni* and the rest were *C. lari*.

TABLE 1 Dairy farms selected for inline milk filter sampling

	Number of collected inline milk filters				
	Small farms (1–49 cows)	Medium farms (50–99 cows)	Large farms (>100 cows)	Unknown size[a]	All farms
Spring sampling period	53	59	26	1	139
Skåne	19	18	10	1	48
Västra Götaland	20	22	5	0	47
Öland	14	19	11	0	44
Autumn sampling period	70	47	43	3	163
Skåne	23	13	13	2	51
Västra Götaland	30	20	9	0	59
Öland	17	14	21	1	53
Total number of samples	123	106	69	4	302

[a]Number of animals not reported by farmers.

TABLE 2 Thermotolerant Campylobacter and STEC from inline milk filters by PCR screening and by isolation

Sampling period	Herd size	Campylobacter, PCR-screening	Campylobacter, isolation	STEC, PCR-screening	STEC, isolation
Spring	1–49	22.6 (12/53)	5.7 (3/53)	37.7 (20/53)	9.5 (6/53)
	50–99	42.4 (25/59)	22.0 (13/59)	74.6 (44/59)	22.0 (13/59)
	>100	53.8 (14/26)	30.8 (8/26)	92.3 (24/26)	26.9 (7/26)
	Unknown	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)
	Total	36.7 (51/139) a	17.3 (24/139) a	63.3 (88/139) a	18.7 (26/139) a
Autumn	1–49	11.4 (8/70)	1.4 (1/70)	61.4 (43/70)	5.7 (4/70)
	50–99	29.8 (14/47)	11.9 (5/42)	80.9 (38/47)	10.6 (5/47)
	>100	39.5 (17/43)	16.3 (7/43)	95.3 (41/43)	16.3 (7/43)
	Unknown	66.7 (2/3)	33.3 (1/3)	100 (3/3)	33.3 (1/3)
	Total	25.2 (41/163) a	8.6 (14/163) a	76.7 (125/163) a	10.4 (17/163) a
Total	1–49	16.3 (20/123) b, c	3.3 (4/123) b	51.2 (63/123) b, c	8.1 (10/123) b, c
	50–99	36.8 (39/106) b	17.0 (18/106) c	77.4 (82/106) b, d	17.0 (18/106) b
	>100	44.9 (31/69) c	21.7 (15/69) b, c	94.2 (65/69) c, d	20.3 (14/69) c
	Unknown	50.0 (2/4)	25.0 (1/4)	75.0 (3/4)	25.0 (1/4)
	Total	30.5 (92/302)	12.6 (38/302)	70.5 (213/302)	14.2 (43/302)

Notes: Results are shown as percent, with total number of positives and number of samples in parenthesis. Within columns same letter (a–d) indicate significant difference ($p < 0.05$). Results for different herd size for spring and autumn sampling period were not statistically analyzed.
Abbreviations: PCR, polymerase chain reaction; STEC, shiga toxin-producing *Escherichia coli*.

A significantly higher proportion of thermotolerant *Campylobacter* was obtained, by PCR-screening and in isolates, in the spring sampling period (Table 2). With PCR screening, thermotolerant *Campylobacter* was found in 36.7% (51/139) of the samples taken in spring compared with 25.2% (41/163) of the samples taken in autumn. The proportion of thermotolerant *Campylobacter* isolated was 17.3% (24/139) in the spring sampling period, compared with 8.6% (14/163) in the autumn. A significantly higher proportion of thermotolerant *Campylobacter* was also isolated from herds with more than 100 animals (Table 2). In addition, a significantly higher proportion of thermotolerant *Campylobacter* was isolated from inline milk filters obtained from farms using untethered milking systems (24/123) compared with tethered milking systems (13/177; Table 3). No significant differences in isolated thermotolerant *Campylobacter* were found when comparing automatic and manual milking systems (Table 3), or when comparing the three sampling regions (data not shown).

3.4 | Shiga toxin-producing *Escherichia coli*

The PCR screening detected *stx* genes (stx_1, stx_2, or both) in 71% (213/302) of the inline milk filters included in the study and STEC was successfully isolated from 14% (43/302) of these (Table 2). The rate of

TABLE 3 Thermotolerant Campylobacter and STEC in relation to the milking system used, by PCR screening and by isolation

Milking system	Campylobacter, PCR-screening	Campylobacter, isolation	STEC, PCR-screening	STEC, isolation
Manual systems[a]	30.2 (73/242)	11.6 (28/242)	66.5 (161/242) a	13.2 (32/242)
Automatic systems[b]	30.5 (18/59)	15.3 (9/59)	86.4 (51/59) a	16.9 (10/59)
Untethered systems[c]	43.5 (54/124) a[d]	19.4 (24/124)[6]	87.1 (108/124) b	17.7 (22/124)
Tethered systems[e]	20.3 (36/177) a	7.3 (13/177)[6]	58.8 (104/177) b	11.3 (20/177)
Milking system unknown	100.0 (1/1)	100.0 (1/1)	100.0 (1/1)	100.0 (1/1)
Total amount of positives	30.5 (92/302)	12.6 (38/302)	70.5 (213/302)	14.6 (44/302)

Note: Results are shown as percent, with total numbers of positives and number of samples in parenthesis.
[a]Manual systems; tube, tethered, pit, untethered, and carousel.
[b]Automatic systems; robot.
[c]Untethered systems; pit, carousel, untethered, robot.
[d]Within columns, and for manual/automatic and untethered systems respectively, the same letter (a–c) indicate a significant difference ($p < 0.05$).
[e]Tethered systems; tube and tethered.
Abbreviations: PCR, polymerase chain reaction; STEC, shiga toxin-producing *Escherichia coli*.

isolation of the PCR-positive samples was 20% (43/213). In total, 45 STEC strains were isolated, as some of the inline milk filters contained more than one STEC strain. Of the isolated STEC, 21 contained the stx_1 gene, 19 the stx_2 gene, and five a combination of both stx_1 and stx_2 genes (Table 4). In total, 0.7% (2/302) of the inline milk filters contained STEC that were positive for both the stx_2 gene and *eae* gene and 5% (14/302) contained STEC that were positive for both stx_1 gene and *eae* gene.

Characterization with whole genome sequencing was performed for 34 of the 45 STEC isolates. The results showed that the isolates belonged to 21 different serotypes, of which STEC O145:H28 was the most common (Table 4). Four of the five isolated STEC O145:H28 (stx_{1a}- and *eae*-positive) were found in milk filters from the Öland region and one was from the Skåne region. STEC O157:H7 was isolated from one of the samples, from a farm in the Skåne region. The STEC O157:H7 isolate contained the stx_{2a} and stx_{2c} genes, but did not belong to the lineage clade 8. In total, 24% (8/34) of the sequenced isolates contained the stx_{2a} gene, 5.9% (2/34) had stx_{2a} in combination with the *eae* gene, 15% (5/34) contained the stx_{2d} gene, and 38% (13/34) had stx_{1a} in combination with the *eae* gene.

A significantly higher proportion of samples tested positive for *stx* genes in PCR screening of inline milk filters sampled in autumn compared with filters sampled in spring (Table 2). However, no such correlation was found for isolated STEC (Table 2). A significantly higher proportion of samples testing positive for *stx* genes was obtained from herds with 50 animals or more (Table 2). The proportion of inline milk filters with successfully isolated STEC was significantly higher on farms with more than 100 milk animals than on farms with less than 100 and less than 50 animals (Table 2). In addition, there was a higher proportion of isolated STEC in samples from farms using untethered milking systems (Table 3).

4 | DISCUSSION

The use of inline milk filters has been confirmed to be a sensitive method for screening of pathogens in milk (Artursson, Schelin, Thisted Lambertz, Hansson, & Olsson Engvall, 2018; Giacometti et al., 2012; van Kessel, Karns, Lombard, & Kopral, 2011). Inline milk filters are designed to catch debris and feces particles, with pore dimensions that do not retain bacteria. Thus, a positive inline milk filter can be interpreted as an indication of contaminated tank milk. However, there are some factors that need to be taken into account when interpreting the results of the present study. First, participation was voluntary for the farms and farms with confirmed *Salmonella* were excluded from the sample set. Second, samples were only collected from three selected regions previously shown to have a high prevalence of STEC O157:H7 and/or *Salmonella* in cattle. Thus, the results should not be taken as representative for the whole country, but rather as reflecting the contamination situation in areas where pathogens are known to be present in the dairy environment.

The prevalence of *Salmonella* is low in Swedish dairy herds and the seroprevalence in bulk tank milk has previously been shown to be 3% (Ågren et al., 2016). However, Ågren et al. (2016) observed regional variations in *Salmonella* occurrence in Sweden and reported >8% herd seroprevalence of *Salmonella* for one of the regions included in the present study, the island of Öland off south-east Sweden. However, no *Salmonella* was isolated from the inline milk filters analyzed in the present study. This could be because of the exclusion of farms with confirmed *Salmonella* or because of reluctance to participate in the study among farmers with a previous history of *Salmonella* in their herds (Artursson et al., 2018). Thermotolerant *Campylobacter* was isolated from 12.6%, of the inline milk filters tested in the present study. This incidence is in agreement with findings reported previously for Swedish dairy milk (Artursson et al., 2018). In Ireland, a survey of raw milk reported detection of *Campylobacter* in 22% of the inline milk filters

TABLE 4 Description of STEC serotypes found in inline milk filters using whole genome sequencing

Serotype	Number of samples	Subtype *stx*[a]	Presence of *eae*
O145:H28	5	stx_{1a}	+
O91:H21	2	stx_{1a}, stx_{2a}, stx_{2d}	–
O91:H21	1	stx_{2d}	–
O26:H11	2	stx_{1a}	+
O154:H31	2	stx_{1d}	–
O182:H25	2	stx_{1a}	+
O182:H25	1	stx_{2a}	+
O136:H12	1	stx_{2a}	–
O136:H12	1	stx_{1a}	–
O81:H21	1	stx_{2c}, stx_{2d}	–
O84:H2	1	stx_{1a}	+
O8:H9	1	stx_{2c}	–
O185:H7	1	stx_{2c}	–
O185:H28	1	stx_{1a}, stx_{2a}	–
O168:H8	1	stx_{2d}	–
O157:H7	1	stx_{2a}, stx_{2c}	+
O113:H4	1	stx_{2d}	–
O109:H48	1	stx_{1a}	–
O109:H16	1	stx_{2a}	–
O103:H2	1	stx_{1a}	+
O103:H2/H45	1	stx_{1a}	+
O5/O8:H9	1	stx_{2c}	–
O5/O2:H27	1	stx_{2a}	–
O8/O22:H8/H19	1	stx_{2e}	–
O.N.T:H28[b]	1	stx_{1a}	+
O.N.T:H19[b]	1	stx_{2a}	–
Total number of isolated STEC	34		

Abbreviation: STEC, shiga toxin-producing *Escherichia coli*.
[a]stx_{2f} cannot be detected with the method used (ISO/TS 13136:2012).
[b]O.N.T serogroup not typable.

tested (FSAI, 2015), while a similar study in Italy isolated *Campylobacter* from 6.4% of inline milk filters (Giacometti et al., 2012).

Stx genes were detected by PCR screening in 71% of the inline milk filters analyzed in the present study. A similarly high proportion has been reported in the United States, with 65% of inline milk filters included in a nationwide survey testing positive for *stx* genes with PCR (van Kessel et al., 2011). In contrast, a survey of milk filters in Italy reported 8.4% prevalence of positive samples for *stx* genes (Giacometti et al., 2012), which is much lower than the level detected in the present study. PCR screening is a very sensitive method for detecting *stx* genes, but has limitations such as lack of distinction between viable or nonviable bacteria, whether the detection is made on free-living phages from the environment, and whether the detection refers to other bacteria containing the *stx* genes (Krüger & Lucchesi, 2015; Martínez-Castillo & Muniesa, 2014).

In this study, STEC was isolated from 14% of the inline milk filters tested. Isolation of STEC from food is generally considered difficult, but isolating STEC from unpasteurized milk is even more challenging due to the high level of background flora (Jackson et al., 2012; Malorny, Löfström, Wagner, Krämer, & Hoorfar, 2008). The isolation frequency of STEC from the positive enrichment broths in the present study was relatively high (20%) compared with that in other studies, in which isolation rates of 9–12.5% have been reported (Giacometti et al., 2012; Jackson et al., 2012; Malorny et al., 2008; Marozzi et al., 2016; Vernozy-Rozand, Montet, Berardin, Bavai, & Beutin, 2005). The use of immunoblotting as an isolation method is a contributing factor to the high-isolation frequency of STEC from *stx*-positive enrichment broths. However, immunoblotting is a laborious technique that cannot be performed easily in routine analyses and is more suitable for surveys where several samples can be analyzed together. When isolation was performed, it was commonly found that only one *stx* gene was detected in each inline milk filter, whereas the PCR analyses often resulted in detection of combinations of more than one *stx* gene. This could indicate that different STEC strains were present in the inline milk filter, but only some of them could be isolated, or that PCR screening detected nonviable bacteria or non-STEC bacteria that possess the *stx* gene/s. Isolation of STEC is thus important to eliminate false positive results from PCR screening and to allow characterization of the STEC isolates, which can be an important factor in risk assessment and risk management (FAO & WHO, 2018). This underlines the importance of improving the methodology for isolation of STEC in food.

Data on the prevalence of STEC in unpasteurized milk in EU are limited, due to the low number of reporting countries (EFSA, 2015). Previous studies have reported absence or low prevalence of STEC O157:H7 in unpasteurized milk or inline milk filters (FSAI, 2015; Artursson et al., 2018; D'Amico et al., 2008; Hill et al., 2012; Oliver, Boor, Murphy, & Murinda, 2009). Similarly, in the present study only one STEC O157:H7 isolate was found, while STEC O145:H28 was the most commonly detected serotype. However, the most common serotypes found in Swedish human cases during 2016 were O26:H11, O103:H2, O157:H7, O145:H28, and O121:H19 (Folkhälsomyndigheten, 2019). Of these, the serotypes O157:H7, O26:H11, and O103:H2 were also found in the present study. Three of the serotypes found in the present study (i.e., O26:H11, O182:H25, and O157:H7) have also been found in Swedish cases that have developed HUS (Folkhälsomyndigheten, 2019).

The toxin Stx_2 is more frequently associated with development of HUS than the toxin Stx_1 (EFSA, 2013; Nataro & Kaper, 1998). With the classification of risk becoming more specific, Stx_{2a} has recently been shown to be associated with severe human illness more often than other subtypes of Stx_2 (FAO & WHO, 2018; Fuller, Pellino, Flagler, Strasser, & Weiss, 2011; Russo, Melton-Celsa, & O'Brien, 2016). In the risk classification model for STEC developed by FAO and WHO (2018), the risk is categorized based on virulence gene content. The highest level of risk (Level 1 of 5), which poses a risk of causing severe disease, is based on detection of the stx_{2a} gene in combination with the *eae* or *aggR* genes. In this study, 5.9% of the sequenced isolates had stx_{2a} in combination with *eae* (level 1) and

15% had stx_{2d} (level 2), which implies a significant risk of causing severe illness.

Seasonal variations have been reported previously for both *Campylobacter* and STEC in cattle. For STEC, studies in Sweden and Scotland have reported higher prevalence in the cooler months of the year, with a possible peak in autumn (Boqvist et al., 2009; Ogden, MacRae, & Strachan, 2004). It has been suggested that the infection is less easily transferred between animals or that the animals rid themselves more easily of the pathogen during the summer grazing period (Boqvist et al., 2009; Jonsson, Aspán, Eriksson, & Vågsholm, 2001). However, others have reported a peak in STEC shedding in the warmer period of the year (Edrington et al., 2004; Ekong, Sanderson, & Cernicchiaro, 2015; Fernández, Rodríguez, Arroyo, Padola, & Parma, 2009). In the present study, no correlation with sampling season was seen for isolated STEC, but a significantly higher proportion of the autumn samples tested positive for *stx* genes. For *Campylobacter*, a peak in bovine shedding has previously been reported to occur during the warmer time of year (Grove-White, Leatherbarrow, Cripps, Diggle, & French, 2010; Nylen et al., 2002). However, in the present study, a significantly higher proportion of thermotolerant *Campylobacter* was detected in the spring samples.

A previous study on STEC O157:H7 in the environment on Swedish farms reported significantly higher prevalence of STEC O157:H7 on farms with a higher number of dairy cows (Widgren et al., 2015). In the United States, a study of bulk tank milk and milk filters reported higher prevalence of *Campylobacter* in dairy units with 100–499 cows and >500 cows, compared with dairy units with 30–99 cows (Del Collo et al., 2017). Similarly, in the present study, a positive correlation was seen between higher number of animals per farm and proportion of isolated *Campylobacter* and STEC in inline milk filters. Data from Statistics Sweden show that there were 320,000 milk-producing cows in Sweden in 2018 and that while the dairy cow population has decreased by approximately 50% over the past 40 years, the proportion of farms with more than 100 animals has increased (SCB, 2018b). As the occurrence of both *Campylobacter* and STEC was found to be higher in inline milk filters from farms with more than 100 animals, this needs to be considered. In addition, the results showing a higher proportion of STEC and *Campylobacter* in filters from farms with untethered milking systems require monitoring, as many farms in Sweden are converting to untethered systems.

5 | CONCLUSIONS

This study showed that both *Campylobacter* and STEC can be present in unpasteurized milk from Swedish farms. Thus, consumption of unpasteurized milk can increase the risk of exposure to foodborne pathogens. This underlines the importance of applying control measures and providing information in order to minimize the risk of infection among raw milk consumers. The most efficient measure to control foodborne pathogens in milk is heat treatment prior to consumption.

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