Norwegian University of Life Sciences
Faculty of Veterinary Medicine
Department of Paraclinical Sciences

Philosophiae Doctor (PhD)
Thesis 2022:71

Norwegian raw cow`s milk, a potential source of zoonotic pathogens?

Norsk rå kumelk, en kilde til zoonotiske patogener?

Lene Idland

Norwegian raw cow`s milk, a potential source of zoonotic pathogens?

Norsk rå kumelk, en kilde til zoonotiske patogener?

Philosophiae Doctor (PhD) Thesis

Lene Idland

Department of Paraclinical Sciences
Faculty of Veterinary Medicine
Norwegian University of Life Sciences

Ås 2022

Thesis number 2022:71
ISSN 1894-6402
ISBN 978-82-575-2024-3

Table of Contents

Acknowledgements

The work on this thesis was performed at the Food Safety Unit, Department of paraclinical sciences and at the Department of Production Animal Clinical Sciences at the Faculty of Veterinary Medicine, Norwegian University of Life Sciences (NMBU) between 2019-2022. The overall aim of this research was to study pathogenic bacteria in Norwegian raw milk, and the project was funded by NMBU.

First, thank you to Toril Lindbäck, my main supervisor, for supporting me through this journey, for encouragement, helpful feedback and being a mental support. Also, a big thank you to my co-supervisors, Marina Aspholm and Erik Georg Granquist, for sharing expertise and guiding me through this project. I am so grateful for the opportunity to complete my doctoral degree with you by my side.

I also want to express sincere gratitude to everyone who have contributed to the progression of my work. Thank you so much to Marte Monshaugen and Kristin O`Sullivan for invaluable guiding in laboratory work, to veterinary nursing students Elínborg Steinunn Pálsdóttir and Henriette Sofie Ross Pedersen for participating in sample collection and to Eystein Skjerve for contributing to statistics and in creating the database. I wish to acknowledge Anette Wold Åsli and Tove Maugesten (Nofima) for welcoming me to use their BSL3 laboratory and for patiently answering all my questions, as well as Mariann Arnyasi and Matthew Peter Kent work (CiGene, NMBU) for teaching me Nanopore sequencing. Thanks to Annette Fagerlund for valuable instructions in WGS analysis.

A special thank you to my colleagues at the Food Safety Unit. Thanks for sharing knowledge, for all the motivation and good lunch talks. To the phd-

students, and especially May Linn Buberg with whom I shared office, I am so thankful for sharing this experience with you. Thanks to family and friends for loving support!

Finally, with all the respect to Norwegian Dairy farmers, thank you so much for your generosity and welcoming me to collect samples at your farms. Thank you for the effort you put in every day to produce safe and high-quality food.

Oslo, October 2022
Lene Idland

1 Abbreviations

A/E	Attaching and effacing
ALOA	Agar Listeria according to Agosti and Ottaviani
AMS	Automatic milking system
BHI	Brain-heart infusion
BTM	Bulk tank milk
CC	Clonal complex
CDC	Centers for Disease Control and Prevention
CFU	Colony-forming unit
CI	Confidence interval
CMS	Conventional milking system
cgMLST	Core genome multi-locus sequence typing
CT	(cgMLST) complex type
DNA	Deoxyribonucleic acid
EHEC	Enterohemorrhagic *Escherichia coli*
EPEC	Enteropathogenic *E. coli*
Eru	EHEC phage replication unit
EU	European Union
FHI	The Norwegian Institution of public health
HUS	Hemolytic uremic syndrome
IMS	Immunomagnetic separation
ISO	International Organization for Standardization
LAB	Lactic acid bacteria
LB	Lysogeny broth
LEE	Locus of Enterocyte Effacement
mCCDA	Modified charcoal cefoperazone deoxycholate agar
MF	Milk filter
MLST	Multi-locus sequence typing
MMC	Mitomycin C

MSIS	Norwegian Surveillance System for Communicable Diseases
mTSB	Modified tryptone soya broth
NMKL	Nordic Committee on Food Analysis
NVI	Norwegian Veterinary Institute
OD	Optical density
ONT	O-antigen nontypeable strain
OR	Odds ratio
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
qRT-PCR	Quantitative real time PCR
SCC	Somatic cell count
Se	Sensitivity of a diagnostic test
SMAC	MacConkey Agar with Sorbitol
SNP	Single nucleotide polymorphism
Sp	Specificity of a diagnostic test
ST	(MLST) sequence type
STEC	Shiga toxin-producing *Escherichia coli*
Stx	Shiga toxin
TBC	Total bacterial count
TBEV	Tick-borne Encephalitis virus
TM	Teat milk
TS	Teat swab
TSB	Trypticase soy broth
UTH	Ultra-high temperature
UPM	Unpasteurized milk
VKM	The Norwegian Scientific Committee for Food and Environment
wgMLST	Whole genome multi-locus sequence typing
wgSNP	Whole genome single-nucleotide polymorphism
WGS	Whole-genome sequencing

2 Summary

The worldwide emerging trend of eating "natural" foods, that has not been processed, also applies for beverages. According to Norwegian legislation, all milk must be pasteurized before commercial sale but drinking milk that has not been heat-treated, is gaining increasing popularity. Scientist are warning against this trend and highlights the risk of contracting disease from milk-borne microorganisms. To examine potential risks associated with drinking unpasteurized milk in Norway, milk- and environmental samples were collected from dairy farms located in south-east of Norway. The samples were analyzed for the presence of specific zoonotic pathogens; *Listeria monocytogenes, Campylobacter* spp., and Shiga toxin-producing *Escherichia coli* (STEC). Cattle are known to be healthy carriers of these pathogens, and *Campylobacter* spp. and STEC have a low infectious dose, meaning that infection can be established by ingesting a low number of bacterial cells. *L. monocytogenes* causes one of the most severe foodborne zoonotic diseases, listeriosis, that has a high fatality rate. All three pathogens have caused milk borne disease outbreaks all over the world, also in Norway.

During this work, we observed that the prevalence of the three examined bacteria were high in the environment at the examined farms. In addition, 7% of the milk filters were contaminated by STEC, 13% by *L. monocytogenes* and 4% by *Campylobacter* spp. Four of the STEC isolates detected were *eae*-positive, which is associated with the capability to cause severe human disease. One of the *eae*-positive STEC isolates were collected from a milk filter, which strongly indicate that Norwegian raw milk may contain potential pathogenic STEC.

To further assess the possibilities of getting ill by STEC after consuming raw milk, we examined the growth of the four *eae*-positive STEC isolates in raw

milk at different temperatures. All four isolates seemed to have ability to multiply in raw milk at 8°C, and one isolate had significant growth after 72 hours. Incubation at 6°C seemed to reduce the number of bacteria during the first 24 hours before cell death stopped. These findings highlight the importance of stable refrigerator temperatures, preferable $\leq$ 4°C, for storage of raw milk.

The *L. monocytogenes* isolates collected during this study show genetic similarities to isolates collected from urban and rural environmental locations, but different clones were predominant in agricultural environments compared to clinical and food environments. However, the results indicate that the same clone can persist in a farm over time, and that milk can be contaminated by *L. monocytogenes* clones present in farm environment.

Despite testing small volumes (25 mL) of milk, we were able to isolate both STEC and *Campylobacter* spp. directly from raw milk. A proportion of 3% of the bulk tank milk and teat milk samples were contaminated by *Campylobacter* spp. and one STEC was isolated from bulk tank milk. *L monocytogenes* was not detected in bulk tank milk, nor in teat milk samples. The agricultural evolvement during the past decades have led to larger production units and new food safety challenges. Dairy cattle production in Norway is in a current transition from tie-stall housing with conventional pipeline milking systems, to modern loose housing systems with robotic milking. The occurrence of the three pathogens in this project were higher in samples collected from farms with loose housing compared to those with tie-stall housing.

Pasteurization of cow's milk is a risk reducing procedure to protect consumers from microbial pathogens and in most EU countries, commercial

distribution of unpasteurized milk is legally restricted. Together, the results presented in this thesis show that the animal housing may influence the level of pathogenic bacteria in the raw milk and that ingestion of Norwegian raw cow's milk may expose consumers to pathogenic bacteria which can cause severe disease, especially in children, elderly and in persons with underlying diseases. The results also highlight the importance of storing raw milk at low temperatures between milking and consumption.

3 Sammendrag (Summary in Norwegian)

Å spise mat som er mindre prosessert og mer «naturlig» er en pågående trend i Norge og i andre deler av verden. Interessen for å drikke melk som ikke er varmebehandlet, såkalt rå melk, er også økende. I Norge er det påbudt å pasteurisere melk før kommersielt salg for å beskytte forbrukeren mot sykdomsfremkallende mikroorganismer. Fagfolk advarer mot å drikke rå melk, og påpeker risikoen for å bli syk av patogene bakterier som kan finnes i melken.

I denne avhandlingen undersøker vi den potensielle risikoen det medfører å drikke upasteurisert melk fra Norge. I tillegg til å samle inn tankmelk- og speneprøver fra melkegårder i sørøst Norge, samlet vi også miljøprøver fra de samme gårdene for å kartlegge forekomst og for å identifisere potensielle mattrygghetsrisikoer i melkeproduksjonen. Alle prøvene ble analysert for de zoonotiske sykdomsfremkallende bakteriene *Listeria monocytogenes, Campylobacter* spp., og Shiga toksin-produserende *Escherichia coli* (STEC). Kyr kan være friske smittebærere av disse bakteriene, som dermed kan etablere et reservoar på gårdene. Bakteriene kan overføres fra gårdsmiljøet til melkekjeden og dermed utfordre mattryggheten. Disse bakteriene har forårsaket melkebårne sykdomsutbrudd over hele verden, også i Norge. *Campylobacter* spp. og STEC har lav infeksiøs dose, som vil si at man kan bli syk selv om man bare inntar et lavt antall bakterieceller. *L. monocytogenes* kan gi sykdommen listeriose, en av de mest alvorlige matbårne zoonotiske sykdommene vi har i den vestlige verden.

Resultater fra denne oppgaven viser en høy forekomst av de tre patogenene i gårdsmiljøet. I tillegg var 7% av melkefiltrene vi testet positive for STEC, 13% positive for *L. monocytogenes* og 4% positive for *Campylobacter* spp.. Fire av

STEC isolatene bar genet for Intimin, *eae*, som er ansett som en viktig virulensfaktor som øker sjansen for alvorlig sykdom. Ett av de *eae*-positive isolatene ble funnet i et melkefilter, noe som indikerer at norsk rå melk kan inneholde patogene STEC. For å videre vurdere risikoen for å bli syk av STEC fra rå melk undersøkte vi hvordan de fire *eae*-positive isolatene vokste i rå melk lagret ved forskjellige temperaturer. For alle isolatene økte antall bakterier etter lagring ved 8°C, og for et isolat var veksten signifikant. Etter lagring ved 6°C ble antallet bakterier redusert de første 24 timene, deretter stoppet reduksjonen i antall bakterier. Disse resultatene viser hvor viktig det er å ha stabil lav lagringstemperatur for rå melk, helst $\leq$ 4°C.

L. monocytogenes isolatene som ble samlet inn fra melkegårdene viste genetiske likheter med isolater samlet inn fra urbane og rurale miljøer rundt omkring i Norge. Derimot var kloner som dominerte i landbruksmiljøet forskjellige fra kliniske isolater og isolater fra matproduksjonslokaler. Videre så man at en klone kan persistere på en gård over tid og at melk kan kontamineres av *L. monocytogenes* kloner som er til stede i gårdsmiljøet.

Til tross for små testvolum av tankmelken (25 mL) fant vi både STEC og *Campylobacter* spp. i melkeprøvene. 3% av tankmelkprøvene og speneprøvene var positive for *Campylobacter* spp. og ett STEC isolat ble funnet i tankmelk. *L. monocytogenes* ble ikke funnet direkte i melkeprøvene. Landbruket i Norge er i stadig utvikling der besetningene blir større, men færre. Melkebesetningene er midt i en overgang der tradisjonell oppstalling med melking på bås byttes ut med løsdriftssystemer og melkeroboter. Forekomsten av de tre patogenene funnet i denne studien var høyere i besetningene med løsdrift sammenliknet med besetningene som hadde melkekyrne oppstallet på bås.

Pasteurisering er et viktig forebyggende tiltak for å beskytte konsumenter fra mikrobielle patogener, og i de fleste EU-land er kommersielt salg av rå melk juridisk begrenset. Denne studien viser at oppstallingstype kan påvirke nivåene av patogene bakterier i gårdsmiljøet og i rå melk. Inntak av rå melk kan eksponere forbruker for patogene bakterier som kan gi alvorlig sykdom, spesielt hos barn, eldre og personer med underliggende sykdommer. Resultatene underbygger viktigheten av å pasteurisere melk for å sikre mattryggheten, og at det er avgjørende å lagre rå melk ved kontinuerlig lave temperaturer for å forebygge vekst av zoonotiske patogener.

4 List of papers

I **The prevalence 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**
Idland L, Granquist EG, Aspholm M, Lindbäck T
Journal of Applied Microbiology, 2022
DOI: 10.1111/jam.15512

II **Whole-Genome Sequencing Analysis of *Listeria monocytogenes* from Rural, Urban, and Farm Environments in Norway: Genetic Diversity, Persistence, and Relation to Clinical and Food Isolates**
Fagerlund A, Idland L, Heir E, Møretrø T, Aspholm M, Lindbäck T, Langsrud S
Applied and Environmental Microbiology, 2022
DOI: 10.1128/aem.02136-21

III **The Ability of Shiga Toxin-Producing *Escherichia coli* to Grow in Raw Cow´s Milk Stored at Low Temperatures**
Idland L, Bø-Granquist EG, Aspholm M, Lindbäck T
Foods, 2022
DOI: 10.3390/foods11213411

5 Introduction

5.1 Bovine milk production in Norway

Milk has been important for the human diet since historical times [1]. Worldwide, cow's milk is nutritionally regarded as a high-quality food. Milk is an important source of minerals, fat and proteins, and in many parts of the world, it is a crucial source of nutrition [1]. The average consumption in Norway is 79 L of drinking milk per person per year (2021) in addition to other dairy products like yoghurt, butter, sour cream, cream, and cheese. In 2021, Norway was self-sufficient with dairy milk from 6925 dairy herds and four main industrial dairy companies [2]. Consumption of milk and milk products has strong foundation in the Norwegian culture, and the development of the Norwegian society has been strongly influenced by agricultural traditions [3-5].

During the last decades, dairy production has undergone dramatic structural changes, both nationally and internationally. In 1992, the first commercial automatic milking systems (AMS) were installed in the Netherlands [6, 7]. From year 2000, AMS and free-stall housing have become more common at Norwegian dairy farms. AMS uses robots to perform the disinfection, pre-milking stimulation and milking routines, and such systems have gradually replaced conventional milking systems (CMS). Approximately 50,000 AMS units are estimated to be in use worldwide in 2020, and the vast majority of them are located in Europe [7]. In 2020, 57% of the milk produced in Norway came from AMS herds [8]. Implementation of milking robots requires cows held in free-stall systems and by 2021, 67% of a total of 213,000 Norwegian dairy cows were held in loose housing [2]. From year 2034, all Norwegian cattle must be held in loose housing barns according to regulations (FOR-2004-04-22-665 §7 and §32). There is a trend towards fewer, but larger herds, and between 2017 and 2021, the number of herds has been reduced by

approximately 1,300 herds [9]. Despite positive consequences of implementing milking robots and free-stall systems such as improved animal welfare and more accurate records of milk quality, little is known about how changes in housing conditions and herd characteristics influence the microbial composition of raw milk.

The Norwegian dairy cow is carefully bred and produce on average 8.204 kg of milk annually [9]. Some Norwegian farms uses Jersey and Holstein-Friesian, but the main dairy breed is the Norwegian Red, known for its good health and fertility traits combined with strong production performances for both health, milk and meat [10-12]. Silage is the main feed used at Norwegian cattle farms. It is harvested in the summer months and stored in sealed bales, silos, or silage pits for later use. The climate and growth seasons are variable across Norway, resulting in regional differences in feed production strategies and feeding regimes for dairy cattle. The cattle are typically dependent on continuous provision of roughage or feed mixes to compensate for the variations in harvest qualities, both during housing as well as during the grazing season. The roughage is almost always combined with concentrates that balance protein and energy requirements according to individual milk yield and feed intake capacity.

5.2 Milk processing and storage

The production of milk for human consumption is strictly controlled in Norway and in the EU (Regulation (EC) No 853/2004). Cows in AMS herds have voluntary but controlled milking, usually 2-4 times a day, while cows in stall barns are milked twice a day. After entering the bulk milk tank through closed pipes, the milk is cooled to 0-4°C until being collected by the milk truck every 2-3 days. Once at the dairy, the milk is tested for the presence of antibiotics, sensory defects, chemical parameters, somatic cell count (SSC) and total bacterial count (TBC). The milk that has passed the tests, is then

separated, standardized, homogenized, pasteurized, and packed in cartons. Raw milk will always contain bacteria and due to its nutritious nature, it is an excellent growth medium for microorganisms [13]. Therefore, an unbroken cooling chain is essential to prevent bacteria from growing [14]. Even slightly abused temperature can cause dramatic changes in bacterial counts and, consequently, influence the quality and safety of the milk. It has for example been shown that the TBC of bulk tank milk (BTM) increases during storage at 6°C [15]. Notably, even low temperature pasteurized milk will contain viable bacteria, primarily spore-formers, although most pathogenic bacteria are killed [16].

In Norway, the most common process for pasteurizing drinking milk is heating to 72°C for 15 seconds, while ultra-pasteurized milk is heated to e.g. 138°C for 2-4 seconds, making it sterile and adapted for storage at room temperature [17]. Pasteurization of milk is widely used to protect consumers from disease caused by consumption of milk infected by pathogenic microorganisms. It was introduced in Europe in the late 1880s [18, 19], and played a huge role in gaining control over the tuberculosis epidemic affecting the US and Europe in the early 1900s, where 10% of the human tuberculosis cases were considered being caused by consumption of raw bovine milk [20]. Pasteurization of milk has also contributed to reducing other serious diseases such as brucellosis, diphtheria, Q-fever, and scarlet fever. In 1953, it became mandatory by Norwegian law to pasteurize milk before commercial sale. This law is still valid, with the only exception being random sale of raw milk directly from farms (FOR-2008-12-22-1624 §21). Commercial distribution of unpasteurized milk is also restricted across Europe [21]. In the rise of the 21st century, the demand for unpasteurized milk (UPM), also known as raw milk, has increased. Some consumers claim that raw milk provides health benefits and better taste. They also want the freedom of choosing unprocessed and organic foods rather than processed products [18, 20, 22]. To satisfy

consumer's demands, raw milk is sold from vending machines in many countries [23]. However, some countries require that such milk is marked with recommendations for boiling before consumption [14]. Also within the agricultural community, there are farmers that want to engage in small-scale sales of unprocessed milk and milk products directly from the farm in order to utilize the farm`s resources [24]. In response to the increasing request for unprocessed milk, regulations on sale of raw milk and raw cream for human consumption are regularly being assessed by Norwegian authorities, despite professional warnings against liberalizing the regulations [25].

5.3 Infectious milk borne disease

Both pasteurized and unpasteurized milk and milk products have caused outbreaks and sporadic human disease all over the world. From early 1900s there were many health hazards associated with raw milk consumption, like typhoid fever (*Salmonella typhi*), scarlet fever and septic sore throat (*Streptococcal* infections), diphtheria (*Corynebacterium diphtheriae*), tuberculosis and shigellosis [26]. After World War II, pasteurization of milk became more regular, resulting in a dramatic decrease in milk-borne diseases. However, after the 1950´s, infections linked to ingestion of raw milk products, like salmonellosis, staphylococcal intoxication, brucellosis, and yersiniosis, were still common [26]. At the end of the 70´s, campylobacteriosis emerged as a health hazard linked to consumption of raw milk and in the 70´s and 80´s, England, Scotland, and Wales had outbreaks of campylobacteriosis caused by consumption of raw milk, affecting more than 4,000 persons. In the same period, unpasteurized milk was suspected to be the vehicle for infectious disease transmission in a number of listeriosis and salmonellosis cases in the US [26]. Notably, disease outbreaks are still regularly linked to dairy products, despite more strict regulations and control of the food production industry in the 21st century. At least 16 foodborne outbreaks in the EU in 2020, were caused by dairy products and the main

agents involved were *Campylobacter*, *Salmonella*, *Staphylococcus* toxins, STEC and *L. monocytogenes* [27]. However, tick-borne Encephalitis virus (TBEV) impacted the statistics with five outbreaks caused by raw sheep- and goat milk [27]. *L. monocytogenes* had the highest case fatality rate (14.2%) among milk-borne outbreak cases in the EU in 2020 [27]. Even though we still experience outbreaks from pasteurized dairy products, studies in the US estimate that consumption of unpasteurized milk products causes 840 times more infectious disease cases than heat-treated products [28].

There have only been a few food-borne outbreaks linked to dairy products in Norway during the recent years. However, despite strict requirements regarding animal husbandry, milk production and sale (Regulation (EC) No 853/2004; LOV-2003-12-19-124), there are still occasional outbreaks; in 2007, 17 hospital cases of listeriosis, linked to consumption of small scale produced Camembert cheese, were reported [29]. The cheese was pasteurized, but later contaminated by the brine where the cheese was matured and preserved. Furthermore, several high school students and kindergarten children contracted campylobacteriosis after drinking raw milk obtained during farm visits in 2013 and 2021, respectively[1]. The Norwegian Scientific Committee for Food Safety and environment (VKM) carried out a risk assessment on consumption of unpasteurized milk in 2006, and they concluded that consumption of raw milk represented an increased risk of disease, and especially highlighted *L. monocytogenes*, *Campylobacter* spp., and STECs as important hazards [17].

5.4 *Listeria monocytogenes*

Listeriosis is a globally spread human infectious disease caused by *L. monocytogenes*. The symptoms vary from mild and sometimes febrile

[1] https://www.matportalen.no/matvaregrupper/tema/melk_og_melkeprodukter/barnehagebarn_syke_av_raa_melk

gastroenteritis to a severe invasive form with septicemia and meningitis. Most listeriosis cases are caused by consumption of foods containing *L. monocytogenes* [30]. A recent study from Sweden showed that *L. monocytogenes* was able to multiply in milk stored at refrigeration temperature [18], indicating that storage of raw milk may increase the risk of listeriosis. Vulnerable groups like immunocompromised individuals, elderly, pregnant and infants are more prone to serious illness with hospitalizations and high mortality, making surveillance of listeriosis and monitoring of *L. monocytogenes* crucial [18]. *L. monocytogenes* is differentiated in 4 distinct genetic lineages, each comprising several serotypes. Serotype 1/2b and 4b in lineage I and 1/2a in lineage II are most associated with human illness, while lineages III and VI rarely are linked to listeriosis [30].

Although relatively rare, listeriosis is one of the most severe food-borne zoonoses in the EU with 1,876 confirmed cases (16 foodborne outbreaks), an occurrence of 0.42 cases per 100,000 individuals, and a fatality rate of 14.2% in 2020. In the same year, 37 human cases were reported in Norway [27]. According to Centers for Disease Control and Prevention (CDC), four multistate listerioses outbreaks occurred in the US in 2016 resulting in seven deaths [31]. The stable trend in number of human listeriosis cases in Europe makes *L. monocytogenes* an economically and socially important pathogen [27]. With an aging population it will probably be an even more relevant zoonosis in the years to come [32, 33]. To counteract this increasing trend, it is important to define risks along food production chains and to apply corresponding preventative measures. This requires knowledge on *L. monocytogenes* epidemiology and contamination routes, as well as a unified nomenclature across borders.

Outbreaks of listeriosis are often associated with ready-to-eat foods, vegetables, soft cheeses, unpasteurized milk, and other dairy products. When

L. monocytogenes has contaminated food-processing environments it may persist for long periods in biofilms on processing equipment or other surfaces. Adequate hygiene practices are, therefore, crucial for safe food products [34, 35]. *L. monocytogenes´* capacity to survive and grow under adverse conditions like high salt, low temperature, modified atmosphere, and low pH makes it difficult to control and to eliminate from food-processing facilities [34, 35]. In addition to food-production plants, *L. monocytogenes* is often found in biofilms at dairy farms where it can persist for several years and cause a continuous contamination pressure on the raw milk [33]. The most common transmission route for contamination by *L. monocytogenes* of milk at the dairy farms are probably poor-quality silage. Survival and growth of *L. monocytogenes* is favored in silage exposed to oxygen and elevated pH level, hence packing density and adequate sealing of silage is important to control *L. monocytogenes* concentrations [36]. The feed itself, or indirect contamination by animal feces, may introduce the pathogen to the housing area and udder surfaces conferring a risk of contaminating the BTM [33]. Biofilm formation in the milking system can also contribute to *L. monocytogenes* in BTM. *Listeria* mastitis is, however, not regarded a common contamination route [37]. Nearly identical genotypes of *L. monocytogenes* can persist on dairy farms for years, but whether this is caused by repetitive reintroduction from outside sources or circulations within the farm is unknown [38]. Whole genome sequencing allows for determination of evolutionary relationships between isolates, opening new possibilities to analyze for potential persistence.

5.5 *Campylobacter* spp.

Since 2005, campylobacteriosis has been the most reported foodborne zoonotic disease in the EU exemplified by 120,946 cases and 45 deaths in 2020 [27]. The disease causes abdominal pain, high fever, and (sometimes bloody) diarrhea and in some cases complications like reactive joint

inflammation and the demyelinating disorder Guillain-Barré syndrome [39]. The main incriminated agents are *C. jejuni* and *C. coli*, which show an emerging trend of antibiotic resistance [40, 41]. *Campylobacter* species are thermotolerant, Gram-negative spiral or curved shaped rods, which grow under microaerobic conditions [40]. Campylobacteriosis show a seasonal trend in developed countries [42] usually peaking during the summer months in Europe [27]. In developing countries, campylobacteriosis show no seasonal trend, probably due to the stabile warm climate [39, 42].

A broad range of domesticated and wild animals and birds are healthy carriers of the pathogen in their intestine [39]. Consumption of poultry meat and non-treated drinking water are the main risk factors for contracting campylobacteriosis, but unpasteurized milk can also be a route of infection [43]. In 2020, 317 foodborne campylobacteriosis outbreaks were reported in the EU, with broiler meat and raw milk being the most common food vehicles [27]. Dairy products caused 65 foodborne outbreaks in the US during 1997-2008 [44], and raw milk caused outbreaks including 11, 99, 148, 12 and 16 clinical cases in Sweden 2014 [45], Utah 2014 [46], the US 2012 [47] and 2016 [48], and the Netherlands 2007 [49], respectively. Hence, raw milk is a relevant source for contracting campylobacteriosis. *Campylobacter* spp. usually does not reproduce in foods but some strains survive in raw milk at refrigeration temperature for 4-6 [50] and even up to 21 days [51].

The infective dose of *C. jejuni* can vary from 500 to 10,000 cells, depending on the strain, the condition of the bacteria, and the susceptibility of the host [52-55]. The occasionally low infectious dose will increase the risk of contracting campylobacteriosis when present in foods.

Despite the important role of ruminants as a reservoir of *Campylobacter* spp., only a few studies are performed on the occurrence of these bacteria in dairy

farm cattle compared to the extensive number of studies carried out in poultry. Norway has a surveillance program for broilers, where 6.1% of 1,893 flocks tested positive in 2020 [56]. In comparison, only 74 cattle were tested, showing a prevalence of 32%. A total of 2,422 human campylobacteriosis cases were reported in Norway in the same year [57].

5.6 Shiga toxin-producing *Escherichia coli* (STEC)

E. coli is an important component of the normal mammalian gut flora, and most strains are considered harmless. However, some variants have pathogenic potential and the pathotype STEC is one of them. STEC is considered an emerging foodborne pathogen known for its potent Shiga-like toxin (Stx), also called verotoxin [58]. In humans, infection with STEC can cause bloody diarrhea and the severe sequelae, hemolytic uremic syndrome (HUS). STECs associated with human disease are referred to as enterohemorrhagic *E. coli* (EHEC). EHEC especially affects young children, where bloody diarrhea is accompanied by HUS in 5-15% of the patients [59]. The serotypes are identified based on their somatic (O) and flagellar (H) antigen [60]. The first large EHEC outbreak described occurred in the US in 1982, and was caused by beef contaminated with the since then well-known *E. coli* of serotype O157:H7 [61]. Following that outbreak, EHEC has become a notable health hazard worldwide, playing an important economical role both in terms of surveillance and treatment costs. Several other serotypes than O157:H7 have also been linked to both sporadic disease cases and outbreaks. Some of the most common known serotypes globally other than O157 are O111, O26, O103, O121, O45, O91, O145 and O146 [27, 62]. O104 became well known after the 2011 outbreak in Germany that caused 845 HUS cases and 54 deaths [62]. Today, EHEC are established as one of the most noteworthy gastrointestinal pathogens, closely monitored worldwide.

STEC produce two main types of the Shiga toxin, Stx1 and Stx2, both further classified in subvariants, of which Stx2a is commonly associated with severe disease [63]. The Stx encoding genes are carried by temperate bacteriophages (Stx phages) which show some similarities to phage lambda [64, 65]. The Stx phages are integrated in the bacterial genome and, during the lysogenic state, both toxin- and phage-production are repressed by the CI repressor [66]. When the CI repressor is removed, spontaneously or after induction, the phage enter the lytic cycle, resulting in production of Stx and new phage particles [67]. Stx is released into the intestinal lumen after phage-mediated lysis of EHEC cells, and it can then be translocated through the epithelial barrier and enter the bloodstream [68]. Once in the blood stream the toxin targets endothelial cells, especially in the kidneys, where it inhibits protein synthesis causing apoptosis that can ultimately result in HUS and renal failure [67]. Stress conditions that may trigger induction of the Stx phages are UV irradiation, oxidative stress and antibiotic treatments. Antibiotic treatment of EHEC infections is therefore generally not recommended [69]. The phage replication unit (Eru) of Stx phages is also believed to impact the virulence of STEC strains [70]. Eru types 1, 2, 5 and 7 have been involved in severe outbreaks, indicating their potential role as virulence factors [70]. Most STEC strains linked to disease outbreaks and HUS also harbor the locus of enterocyte effacement (LEE), encoding intimin (*eae*) and the intimin receptor Tir (*tir*), that participate in causing the attaching and effacing (A/E) lesions in the large intestine epithelium [68, 71]. Although *eae* and *tir* are highly important to EHECs pathogenicity, STECs lacking the *eae* gene have also caused severe outbreaks. For example, the causative strain of the 2011 German outbreak, lacks the LEE locus but carries the locus of "Adhesion and Autoaggregation" encoding another adherence mechanism [72].

Even though not all STECs are pathogenic to humans [58], several outbreaks occur globally every year. In 2020, EU reported 4,446 human clinical EHEC

cases and 34 foodborne outbreaks of disease caused by STEC [27]. A total of 331 human cases of EHEC infections were registered in Norway in 2020, a lower number compared to the year before, after it had increased each year since 2000 [57]. Several dairy-borne outbreaks have been reported in the EU and in North-America between 2000 and 2019 (table 1), mainly caused by the serotypes O157, O92, O145 and O26 [14]. Other serotypes associated with dairy products in the EU are O103, O146, O111, O113 and O126 [14]. As the infectious dose of EHEC is assumed to be very low (<100 bacteria) the risk of becoming infected after consumption of STEC contaminated raw milk is highly relevant [73, 74].

Table 1: Selected EHEC outbreaks associated with milk and dairy products in Europe and the US.

Year	Country	Serotype	Source	Number of cases	Reference
2000	UK	O157	Raw milk	2	[75]
2003	Austria	O26	Raw milk	2	[76]
2003	Slovakia	O157	Raw milk	9	[77]
2003-04	Denmark	O157	Organic milk	25	[78]
2006	USA	O157:H7	Raw milk	2 HUS	[79]
2007	Belgium	O145 and O26	Ice cream from pasteurized milk	12	[80]
2010	USA	O157:H7	Raw milk cheese	8	[58, 81]
2013	Italy	O26:H11	Linked to dairy plants	20	[82]
2017	England	O157:H7	Raw milk	7	[83]
2019	France	O26	Raw cow`s milk soft cheese	16	[84]

Some STEC serotypes can grow at temperatures down to 6.5°C [85] and its ability to grow in food depends on the composition of the substrate. EHEC of serotype O157 has been shown to survive, and even grow, in milk stored at abusive refrigerator temperatures [23, 85-87]. This is a concern considering

its low infectious dose. Raw milk is not a consistent substrate as the microflora can vary, and may thus impact the growth of STEC differently, probably explaining at least part of the differences in growth observed in different studies [88]. One study showed that the number of EHEC slightly decreases in raw milk stored at 4°C over a period of four days [23]. However, another study, showed some growth of EHEC O157:H7 in whole milk (generation time 0.89 d) within the first 24 hours of storage at 4°C. In the same study, nonpathogenic *E. coli* strains grew slightly at 4°C in whole, skimmed, and semi-skimmed milk over the first 4 days of storage [86]. It has also been reported that EHEC O157:H7 and nonpathogenic *E. coli* strains decrease in number over 85 days in milk stored at 4°C and 20°C [86]. Yet another study showed that STEC O157 grew in raw milk stored at 7 and 15°C [87], and that some STEC strains had a generation time of 11 hours in whole milk stored at 9.5°C [85].

5.7 Pathogen prevalence in dairy farms

The dairy farm environment is a common reservoir for zoonotic microorganisms. Cattle can be asymptomatic carriers of several human pathogenic bacteria, and fecal shredding causes widespread contamination in husbandry environments [89]. Known pathogens like *Campylobacter* spp., *L. monocytogenes*, *Salmonella* spp., Shiga toxin-producing *E. coli* (STEC), *Staphylococcus aureus*, *Streptococcus* spp., and *Yersinia* have been reported to have reservoirs in farms [90], several of them have also been detected in milking systems. Pathogenic microbes can be introduced to animal houses through roughage and activities like harvest and livestock trade which, in turn, increases the risk of further transmission to the milk production chain. Other routes for entering the milking system can be through udder infection, teat wounds or from human skin during milking [89].

BTM collected in Ontario 1997 had a prevalence of 2.73%, 0.17%, 0.47% and 0.87% for *L. monocytogenes*, *Salmonella*, *Campylobacter* and STEC,

respectively [91]. BTM filters collected from 58 dairy farms in Sweden in 2017, had a prevalence of 71% for *S. aureus*, 21% for *Listeria* spp. 9% for *C. jejuni*, and 2% for *Y. enterocolitica* and STEC O157 [90]. Of milk filters collected from Finnish farms, 2%, <1% and 29% were positive for STEC O157:H7, *Campylobacter* [73] and *L. monocytogenes*, respectively, and 13% of BTM samples were positive for *L. monocytogenes* [33]. Several studies have reported the presence of *L. monocytogenes* in raw cattle milk: 13% of raw milk samples were positive in a Finnish study [33], 6.3% in a study from Belgium [92], 2.8% in one from the US [93], 5.1% in one from India [94], 6.1% in one from Spain [95] and 1% in a Swedish study [96].

Cattle are often healthy carriers of *Campylobacter* spp. and intermittent shedding in feces can contaminate udders and transfer the bacterium to milk [97]. Previous studies from Finland and Spain have shown that 53% [73] and 81.2% [41] of cattle fecal samples were positive for *Campylobacter*, respectively.

STEC have several reservoirs and they occur frequently in agricultural environments. Recent studies report a high STEC prevalence in monogastric farm animals, companion- and wildlife animals, birds, rodents, in aquaculture and in some insects. However, the gut of ruminants is regarded the most important reservoir for STEC [62]. Dairy cattle, and especially post-weaning calves and heifers, can be asymptomatic carriers of STEC; hence the bacteria can be widespread in the dairy farm environment. In 2014, the Norwegian Veterinary Institute (NVI) conducted a survey on STEC in Norwegian cattle where they isolated STEC belonging to the serotypes O26, O91, O103, O121, O145 and O157 from 15.6% of 179 tested herds [98]. In another study, NVI tested 308 ground meat products from grocery stores for the presence of the same serotypes and detected O26 and O91 in one sample each [57]. In a Finnish study, *stx*-genes were detected by PCR in 37% of milk filters and 7%

of BTM samples, and screening of cattle showed a STEC O157:H7 prevalence of 17% [73]. Furthermore, 1.0% and 0.6% of 25 mL bulk tank milk samples were positive for O157 in two studies from New Zealand [99, 100]. The farm reservoirs explain why contaminated food, animal contact or water contaminated by animal feces are common routes of human STEC infections.

Since *Salmonella* spp. are continuously monitored by NVI, they are not included in this thesis work. However, it has been estimated that *Salmonella* spp. are present in 0.1% in the Norwegian cattle population [101]. The high prevalence of different zoonotic pathogens at farms highlights the importance of pasteurization to ensure production of safe drinking milk.

5.8 The contribution of genomics in pathogen surveillance

The international trade of foods and the high frequency travel increases the risk of transmission of infectious diseases and enables multi country disease outbreaks. This highlights the importance of common nomenclature and efficient and coordinated surveillance systems between countries to rapidly detect outbreaks and find their source. Still, pathogen surveillance is mostly organized on national levels, limiting rapid detection of cross border transmission [102]. However, high throughput sequencing technologies have revolutionized the field of whole genome analyses, allowing for detailed isolate comparison across geographical sites and time. The rapid advances in bioinformatic methods make sequence analysis more accessible and it opens new possibilities to investigate evolutionary relationships, genetic divergence, as well as characteristics of pathogens. The possibility to analyze the entire genome of pathogenic microbes represent a huge advantage in outbreak analysis, as identification of a common source can be done faster and more reliable, also on an international level.

Typing methods used in outbreak analyses to identify the source of the outbreak are continuously changing. Pulsed-field gel electrophoresis (PFGE) has been the golden standard for microbial typing for many pathogens [102]. However, PFGE does not reveal phylogenetic relationships, and it lacks discriminative power to precisely identify common source clusters. DNA sequencing methods can differentiate strains into subtypes based on common genetic characteristics following shared ancestry [30]. Multi Locus Sequence Typing (MLST) is based on comparing the DNA sequence of usually six or seven housekeeping genes to differentiate bacterial strains into clonal complexes (CCs or clones) and sequence types (STs). Core genome MLST (cgMLST) extends the MLST concept to include over a thousand loci to differentiate isolates into cgMLST complex types (CTs) [102]. The Norwegian institute of public health combine MLST and cgMLST in epidemiological surveillance of STEC [103], *L. monocytogenes* [104, 105] and *Campylobacter* [106]. STECs and *L. monocytogenes* are in addition analyzed for serotype, and STEC also for virulence genes [103-105]. Even though MLST and cgMLST improve the identifications of phylogenetic clusters, these methods still lack discriminative power to distinguish common source isolates. Whole genome sequencing (WGS) has been shown to further improve outbreak detection analyses, also in outbreaks associated with raw milk [83, 107]. Whole genome MLST (wgMLST) is currently implemented in foodborne disease surveillance in many countries. Its high taxonomic resolution will hopefully improve comparison of data between laboratories and facilitate international collaboration. In addition to including only loci that are present in all isolates of a given population, as cgMLST does, wgMLST also includes accessory loci which allows for gene-by-gene comparison of isolates [108]. By including a larger percentage of the genome in the analysis, isolates can be clustered with higher discriminative power.

5.9 Knowledge gaps

Findings of zoonotic bacteria in unpasteurized milk from Finland and Sweden [18, 33, 73, 90] together with limited information about the prevalence of these bacteria in Norwegian dairy farms, call for more knowledge about the situation in Norway. Rapid developments in agricultural technologies, together with pathogens ability to rapidly adapt to their surrounding environments, require updated data to identify potential risks linked to consumption of raw milk. Today's agricultural trend leads towards lager production units and more economically profitable operation systems such as automatic milking systems. Does the modern loose housing affect the prevalence of zoonotic bacteria in cow milk? There is also limited data available on the distribution and persistence of zoonotic bacteria in dairy farm and surrounding environments, as well as on their ability to survive and grow in raw milk. Altogether, this thesis provides important information regarding the safety of consumption of unpasteurized milk in Norway.

6 Aims and objectives

The overall aim of this thesis was to study the occurrence of zoonotic agents in raw milk from Norwegian dairy cattle and to analyze how factors at the farm, in the surrounding environment and in stored raw milk influence their presence, persistence and growth. The thesis focuses on *Campylobacte*r, *L. monocytogenes* and STEC since they are among the most relevant zoonoses in Norway and regularly cause milk-borne disease outbreaks worldwide [27]. The results from this work can contribute to the assessment of raw milk consumption as a potential health risk.

The aim was approached through the following objectives:

1. Mapping the prevalence of *Campylobacter* spp., *L. monocytogenes* and STEC in Norwegian dairy farms to attain herd-level prevalence estimates and comparing those between herds with loose housing versus tie-stall housing systems, and between different seasons. Collecting hygiene data from Norwegian dairy cattle herds and relate these data to the presence of *Campylobacter* spp., *L. monocytogenes* and STEC in environment and in raw milk (Paper I).
2. Use comparative genomics to investigate infection routes, potential persistence, and risk factors for increased prevalence of *L. monocytogenes* in Norwegian raw milk. Perform WGS analysis of dairy farm *L. monocytogenes* isolates and compare their sequences to those of isolates from rural and urban environments in Norway, as well as those of clinical and food isolates available in databases (Paper II).
3. Study characteristics of STEC isolated at Norwegian dairy farms to evaluate the health risk associated with consumption of raw milk. Perform cultivation experiments on the above-mentioned isolates to

test how different storage temperatures affect survival and growth STEC in raw milk (Paper III).

7 Summary of papers

Paper I: The prevalence 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

Idland, L., Granquist, E.G., Aspholm, M. and Lindbäck, T.
Journal of Applied Microbiology (2022) 132(5):3959-3972.

The aim of the study was to map the occurrence of *Campylobacter* spp., *L. monocytogenes* and STEC in BTM and in the environment at Norwegian dairy farms to highlight a possible link between herd management and the presence of zoonotic bacteria. It was also investigated how seasonal variations and hygiene management practices influence the presence of pathogenic bacteria.

A total of 18 dairy herds, seven with AMS and free-stalls, nine with CMS and tie-stalls, one free-stalled with a milking carousel and one free-stalled with a milking parlor, were recruited to the study. The farms were located at different geographical regions in the south-east of Norway (Nedre Eggedal, Hokksund, Hadeland, Blaker, Mysen and Rakkestad). All farms were visited six times over a period of 11 months (August 2019 to July 2020). Samples of BTM, milk filters, teat swabs, feces, and feed (silage or silage mixture) were collected at each visit, and teat milk samples were included from visit number three. Hygiene scorings were performed on 30% of the dairy cows during each visit. The samples were analyzed within six hours after collection using standardized reference methods according to International Organization for Standardization (ISO) or Nordic Committee on Food Analysis (NMKL). *L. monocytogenes* was isolated from milk filters (13%), feces (30%), feed (32%) and teat swabs (5%) and *Campylobacter* spp. were found in BTM (3%), milk

filters (4%), feces (68%), teat swab (13%) and teat milk (3%). BTM, milk filters and feces were examined for presence of *stx* and *eae* using PCR. The *stx1* gene was detected in 10% of the BTM samples, and in 9% and 19% of the milk filters and fecal samples. The corresponding results for *stx2* were 10%, 25% and 33%, and for *eae* 15%, 24% and 14%. All samples PCR positive for *stx* or *eae* were plated at CHROMagar STEC and recovered colonies with typical appearance were examined for the presence of *stx* and *eae*. A total of 19 colonies were *stx* positive and, therefore, regarded as STECs (one isolate from BTM, seven from milk filter and 11 from feces). Four of these isolates were double positive for *stx* and *eae* and were, therefore, considered potential high-risk isolates (three fecal and one milk filter isolate). Multiplex PCR were used to determine the O serotype, however, none of the STEC isolates were of the common serotypes O26, O45, O103, O111, O121, O145, O157, nor of serotype O5, O15, O55, O91, O104, O111, O113, O118, O123, O128, O146, O165, O172 or O177.

Regarding *Campylobacter* spp., there was a higher occurrence in feces ($P < 0.01$) and teat swab ($P = 0.03$) from loose housing herds compared to tie-stall herds, and for *L. monocytogenes* in feces ($P = 0.02$) and feed ($P = 0.03$). The high-risk *eae* and *stx* positive *E. coli* isolates as well as the BTM STEC isolate were all detected in loose housed herds.

The study showed a positive association between dairy cow hygiene score and detection of *Campylobacter* spp. in teat milk ($P = 0.03$), and a putative correlation between the dairy cow hygiene score and the presence of *Campylobacter* spp. in milk filters ($P = 0.06$). *L. monocytogenes* were more often detected in milk filters when feces ($P < 0.01$) or feed ($P < 0.01$) were simultaneously positive, indicating that pathogens in the nearby environment increase the chance of also finding them in the milking system.

Seasonal variation in pathogen occurrence were seen for some sample types. Feed was more often positive for *L. monocytogenes* in January compared to the samples collected in June, August and September (P = 0.03). *Campylobacter* spp. were more often detected in feces in November-December and May compared to August-September (P = 0.02 and P = 0.04) and June (P = 0.05 and P = 0.02). Feces positive for *stx*2 were more common in August-September compared to May (P = 0.01) and June (P = 0.02). Contrastingly, there was a trend towards a higher prevalence of STEC in milk filters in May compared to August-September (P = 0.05).

To sum up, this study detected a wide distribution of *L. monocytogenes*, *Campylobacter* spp. and STECs in farm environments highlighting the risk of contaminating the raw milk produced at the farms. New farm technologies may create novel niches for microbes to survive or grow in which can cause food safety challenges. Good hygienic measures seem to reduce the risk of zoonotic pathogens entering the milk production chain.

Paper II: Whole-Genome Sequencing Analysis of *Listeria monocytogenes* from Rural, Urban, and Farm Environments in Norway: Genetic Diversity, Persistence, and Relation to Clinical and Food Isolates

Fagerlund, A.[#], Idland, L.[#], Heir, E., Møretrø, T., Aspholm, M., Lindbäck, T and Langsrud, S.

Applied and Environmental Microbiology (2022) 88(6):e02136-21.

[#] The authors contributed equally

In this study, comparative genomics were used to investigate infection routes, potential persistence, and risk factors for increased prevalence of *L. monocytogenes* in Norwegian raw milk. Whole genome Illumina sequencing was performed on 79 *L. monocytogenes* isolates collected from 18 different dairy herds in the south-east of Norway. Genomic MLST analysis was performed on these isolates and on 115 isolates collected from rural and urban environments across Norway, 24 isolates from slugs, as well as on publicly available genomes of clinical *L. monocytogenes* isolates, to enlighten species diversity and persistence in different environments. The prevalence of *L. monocytogenes* was higher in agricultural and urban areas compared to locations less habited by humans and animals, like forests and mountains. *L. monocytogenes* was found to persist over time in different environments, as samples collected from the same source during different sampling occasions clustered with only 0-8 wgMLST allelic differences.

In the dairy farms, 33 of the 79 *L. monocytogenes* isolates were a part of 12 different clusters with pairwise genetic distances ranging from 0 to 11 wgMLST alleles. Each cluster contained two to four isolates. The isolates within a cluster were collected during repeated samplings at the same farm over a timespan of two to 10 months. A large proportion (94%) of the dairy farm isolates belonged to lineage II, and the predominating clonal complexes

(CCs) were CC11/ST451 (18%), CC91 (15%), and CC37 (11%). These CCs were distinct from those dominating among clinical isolates. CC9 is frequently detected in meat processing environments in Norway [109], but none of the farm isolates in our study were of CC9.

Four out of 12 isolates collected from on-farm milk filters were part of a persistent cluster (CC11, CC91, CC18 and CC177) found on the same farm on multiple visits. These milk filter isolates had 0-7 wgMLST allelic differences compared to isolates from feces, feed, and teat swabs, indicating cross-contamination events. At four occasions, isolates with 9-20 pairwise wgMLST or 0-1 cgMLST allelic differences (CC11, CC226, CC415) were present on distinct farms. There were also six clusters that contained isolates from both dairy farms, and rural and urban environments, with the range of 9-27 wgMLST allelic differences (0-1 cgMLST differences). Two CC37 isolates from feed and teat swabs from a farm 50 km east of Ås had 9 to 14 wgMLST allelic differences to two CC37 isolates collected from grazing land in Ås. CC7, CC1, and the hypovirulent CC121 dominated among clinical *L. monocytogenes* isolates. Nine clusters of isolates collected at different timepoints from different habitats and geographical locations contained clinical isolates. Importantly, definitions often used to identify outbreak clusters, would in this study cluster several apparently unrelated samples.

This study indicated that the same clone can persist in a cattle herd over time, and that clones detected in the farm environment can contaminate milk filters and eventually BTM. The same *L. monocytogenes* clone were also collected from farms located in different geographical regions. *L. monocytogenes* is ubiquitous in natural, urban, and agricultural areas, hence, coordinated surveillance is of great importance to reduce the risk of *L. monocytogenes* contaminated dairy products and other foods.

Paper III: The Ability of Shiga Toxin-Producing *Escherichia coli* to Grow in Raw Cow`s Milk Stored at Low Temperatures

Idland, L., Bø-Granquist, E.G., Aspholm, M., and Lindbäck T.
Foods (2022) 11(21):3411

Knowledge on how temperature abuse affects growth and survival of pathogenic microorganisms in raw milk during storage is needed to suggest measures that can prevent milk borne infections. Objective III was to study growth and survival of STEC in raw milk and to assess risk factors related to consumer handling and storage of raw milk. Four *eae*-positive STEC isolates, collected at Norwegian dairy farms, were subjected to Nanopore sequencing, revealing that the genomes of three out of four isolates were nearly identical. Two of the identical isolates were from the same farm and collected from a milk filter and from a fecal sample during the same sampling occasion. The third identical isolate was, on the other hand, isolated seven months prior to the other two isolates. It was collected from a fecal sample at a farm located approximately 10 km from the first farm. The three isolates carried the genes encoding γ-intimin and Stx1. The fourth isolate was collected from the first farm, but it showed a genetic profile that differed from the other two isolates collected at the same farm. This isolate carried the genes encoding α-intimin, and Stx2. Stx production were inducible with Mitomycin C in all four isolates, indicating that treatment of a potential infection caused by these strains with certain antibiotics could result in increased toxin production.

A storage experiment demonstrated that STEC isolates from Norwegian dairy farms were able to multiply in raw milk at low temperatures. STEC inoculated in raw milk and stored at 4°C decreased in number during the storage time of 72 hours. The reduction was only significant for one isolate. At 6°C, the number of STEC decreased at first, but the cell death seemed to stop after 24

hours. At 8°C, all four isolates propagated during storage, however, the growth after 72 hours was only significant for one isolate. To mimic consumers handling practice by leaving the milk at room temperatures during a meal, some samples were incubated at 20°C for 1.5 hours each day and the rest of the time they were stored at 4°C. All four STECs multiplied under these conditions, however, the growth was only significant for one isolate. The study highlights the importance of continuous cooling of raw milk ≤ 4°C, also during transport. Storage at abused temperature may lead to rapid propagation of STECs which increase the risk of milk borne infections. Storage at low temperatures is especially important regarding STEC, as this pathogen may cause disease at very low doses (<100 bacteria).

8 Material and methodological considerations

Paper I

Recruiting participants to the study

To examine characteristics and occurrence of zoonotic pathogens in raw milk from farms with different operational systems, this study included dairy farms with different herd management. Originally, the idea was to find relevant herds through dairy companies. However, due to strict privacy regulations, this proved to be difficult. We ended up using "Brønnøysundregisteret", where all Norwegian companies and most dairy farms are listed with contact information, to find participants for the project. Farms in the south-east area of Norway, located within 100 km from Oslo, were contacted. As Norway is an elongated country, and cattle husbandry varies across geographical locations, the selected farms may not represent the climate and fauna conditions from all parts of Norway, hence our target population is dairy farms in south-east Norway. A total of 18 farms were recruited to participate in the project as our study sample. Seven of the recruited farms had CMS and tie-stall, whereas nine had AMS and loose housing. The remaining two farms were free stalled herds holding milking carousel and a milking parlor. When looking at differences in pathogen occurrence using AMS versus CMS, and in loose housing versus tie-stall, there is a lot of confounding factors. In general, the herd sizes are larger in free stalled herds, which most likely affect the microbial situation at the farm. It was therefore difficult to conclude if it was the loose housing, herd size or AMS that caused the differences in pathogen occurrence. Many of the farms with loose housing are also more modern, built after a recent legislation demanding all cattle to be free stalled within 2034 (FOR-2004-04-22-665 §7 and §32). The new legislation has led to a new era in Norwegian agriculture,

where many farmers have had to invest a lot of money into renovation of their farms to meet the new management demands. Most often, loose housing herds have automatic feed and manure handling systems in addition to the AMS, which is a costly affair for the farmers to install. For small production units the cost is too high and for the past years the total number of dairy farms in Norway have, therefore, been reduced [5], resulting in larger, but fewer, dairy production herds. Several of the loose housed herds in this study had relatively new farm buildings, which may have affected the pathogen occurrence. Also, the probability of a farmer being willing to make their herd available for a research study may increase when the farm exhibit good management routines, which may result in a group of higher average performance than the true Norwegian dairy farm average.

Sample collection

Samples of BTM, milk filters, feces, feed, teats, and teat milk were collected from each of the 18 dairy herds included in the study. To make the collection of samples more efficient, all farms located in the same region were sampled the same day when possible. Separate bags of equipment were packed for each farm, and only the relevant bag was brought into the animal houses. A cooling box containing freezing elements, for storage of samples, were left outside the biosecurity barrier to avoid contamination between the different farms. Performing sterile sample collection at dairy farms have many sources of errors. A common difficulty was to find clean places to put the sampling gear within a proximity to the working area when sampling. In addition, working with live animals cause unexpected situations like anxious cows acting out, sudden cattle defecation that splashes onto the sampling gear or curious animals approaching the sampling gear or sampler. To maintain good hygiene during sampling, thorough preparations were performed before starting each sample collection, including planning the whole procedure with sterile placement and handling of sampling equipment. If samples were unfortunately contaminated or affected in a way that could affect the result,

the samples were left out of the study and the sampling was repeated if possible. On a few occasions, teat swab, teat milk or fecal samples could not be collected due to animals pastured too far away from the farm. Also, the milk collection truck had sometimes visited the farm prior to the visit, leaving the bulk tank empty or only containing milk from some of the dairy cattle, not necessarily representative of the whole herd. As a result, the dataset does not contain six full samplings from all farms.

BTM samples were collected in sterile Falcon tubes or 1 L autoclaved glass bottles. Fifteen of the farms had a tap connected to the tank, and sample milk were directly drained into the sample containers. The tap and bulk tank could be a location of microbial accumulation if not washed and disinfected properly. The bulk tank components are routinely washed after each milk pickup, using a combination of high temperature water and washing detergents to remove milk soils, organic and mineral solids, as well as residual microorganisms on surfaces. In addition, the tap is flushed after use. Three of the farms had tanks with an opening at the top, and samples were then collected using sterile metal ladles. The ladle was an extra tool needed to be handled in a strictly hygienic way. Prior to samplings, it was packed tightly and autoclaved to avoid contamination.

One milk filter was collected at each sampling visit. The farmers change the milking system filters regularly, at specific times of the day, and sampling had to be performed at that specific time. Clean disposable gloves were used when handling the filters and they were deposited in sterile stomacher bags. The farmers were taught to change the filters aseptically and store them chilled in cases when we were not present, however, the quality of this process could not be assured. Poor communication with the farmers sometimes led to changing of the milk filters without following the guidelines, thereby leaving the filter useless and excluded from the study. After collecting

the milk filters, they were immediately cut longitudinally into three pieces and placed in sterile glass bottles containing mTSB, Bolton broth and Half Fraser broth, respectively. This was done to optimize the conditions for bacterial survival, like avoid drying and extensive air access. Other studies [110] have shown better survival of *Campylobacter* spp. in stool samples when modified Cary-Blair was used as transporter medium, however, we did not employ Cary-Blair broth due to logistic difficulties. Including modified Cary-Blair broth in the study may have ensured an even better survival of at least *Campylobacter* spp..

Approximately 100 g feces were collected using clean disposable gloves and sterile stomacher bags. By collecting feces directly from the cow colon, we would have ensured the sample to be fresh, but for practical reasons and animal welfare, the feces samples were collected from the floor. To ensure collection of representative fecal samples, fecal material was collected from at least five distinct locations in the animal pen and, if possible, at locations with continuously high animal density, like outside the milking robot, feeding- or crossing areas.

Feed samples consisting of silage or silage mixture were collected from the feed tray. A total of approximately 100 g were collected from five to 10 different locations and stored in a sterile stomacher bag until analysis. If the feed tray was empty, the sample were collected from the feed batch about to being served to the cows.

Teat swabs and teat milk from at least 10% of the dairy cows were collected at each farm at each visit. A swab moistened in peptone water were used to rub the surface of all four quarters if possible. Sometimes only two to three teats were swabbed due to practical difficulties of swabbing more, like the cow kicking or not standing still as we did not have time to fixate the cows

before sampling. Due to lack of resources to analyze more samples, swabs from five to 10 cows were pooled to a common sample representing the herd. Teat milk samples were included from the third round of sampling, as a tight time schedule the first two samplings restricted the number of sample types collected. Optimally, teat milk would have been included at the two first samplings as well, to get a complete dataset. Teat milk was collected from the cows that had been swabbed. All four quarters were disinfected after swabbing, and hand milked into Falcon tubes. These samples were also pooled into a common sample representing the herd. During milking, there is always a risk of the sample being contaminated by microbes from the human or animal skin and from dust in the air. To avoid this, small sample containers were used, and disposable gloves were worn if handwash could not be done prior to milking.

Because of the Covid-19 pandemic, there was a pause in sample collection from March to May 2020. The last two samplings, performed in May and June 2020, were not completed at all the farms because of Covid-19 restrictions, leaving a less complete dataset from this period.

A limitation of the study was the small volume of sample material being analyzed. For BTM analysis, 1 L of milk was collected from each farm, and only 25 mL of the total volume was transferred to enrichment broth. To analyze 25 mL from tanks containing several thousand liters of milk may provide an incomplete picture of the situation in the whole tank. The tanks exhibit stirring mechanisms to avoid separation of milk, so the content should be evenly distributed, however, the huge dilution effect may result in difficulties in detecting microorganisms present at low concentrations. In retrospect, analysis of larger volumes would have generated results that better represented the BTM. Only 10 g of feces and feed, and 5 mL of teat milk and teat swab solution were analyzed, due to time and cost limitations.

Culturing samples

Samples were examined for *L. monocytogenes* according to the NMKL method No 136, 5th ed. 2010, used for detection and enumeration of *L. monocytogenes* in food and foodstuffs. *Campylobacter* were assessed according to NMKL No. 119, 3. Ed., 2007, intended for qualitative and quantitative determination of thermotolerant *Campylobacter* in foods and drinking water. For quantification of *L. monocytogenes* and *Campylobacter* spp. 100 µl of the milk samples were plated directly on ALOA (Agar Listeria according to Agosti and Ottaviani) and mCCDA (Modified charcoal cefoperazone deoxycholate agar), respectively. For qualitative detection, the samples were enriched in specific broths to enhance detection of low concentrations and/or weakened bacteria. Due to scarce of both time and economical resources, the direct quantification and qualitative analyses were performed on one single plate each.

Detection of STEC

After enrichment of BTM, milk filter, and fecal-samples in mTSB supplemented with novobiocin according to ISO/TS 13136:2012, DNA were isolated from the samples using DNeasy® Blood and Tissue kit (Quiagen, Hilden, Germany). Isolated DNA were then examined by PCR for the presence of *stx* and *eae*. ISO/TS 13136:2012 uses serotype-specific enrichment (immunomagnetic separation) on samples PCR positive for *stx* or *eae* to enrich for *E. coli* of serotypes O157, O111, O26, O103 and O145. To ensure inclusion of all serotypes, we did not perform immunomagnetic separation. However, all PCR positive samples (either *stx1, stx2* and/or *eae*) were directly plated onto selective agar plates CHROMagar™ STEC (Kanto Chemical Co, Japan) on which STEC grows with characteristic mauve colonies. Three mauve colonies from each CHROMagar plate (regardless of the number) were replated on Sorbitol-MacConkey (SMAC) agar plates. This was done to

identify putative *E. coli* O157:H7 as *E. coli* of this serotype rarely ferment sorbitol and therefore appear with colorless colonies on SMAC agar. The three mauve colonies from the CHROMagar STEC plates were also subjected to PCR using primers directed against *stx*. When positive for *stx*, the isolates were further examined for the presence of *eae* by PCR. By only analyzing three mauve colonies from each plate regardless of the number of mauve colonies, the number of reported STEC may be underestimated.

More than 150 non-O157 EHEC O serogroups have so far been described [111] and to determine the O serotype of *stx* positive colonies appearing mauve on the Chromagar plates, the DNA of the isolates were subjected to multiplex PCR including primers detecting 20 of the most common serotypes [112]. The PCR results indicated that the STEC isolates did not belong to any of the 20 O serotypes tested (O5, O91, O26, O103, O145, O121, O111, O55, O128, O113, O146, O45, O177, O157, O15, O104, O118, O123, O165, O172). Unfortunately, we were no able to include positive controls for other serotypes than O157 and O103, hence we cannot totally exclude the chance that some of the isolates belong to one of the mentioned serotypes.

Paper II

The aim of this paper was to use comparative genomics to investigate contamination routes and potential persistence of *L. monocytogenes* at Norwegian dairy farms. To compare the farm isolates to isolates from rural and urban environments in Norway a collaboration was established with the Norwegian food research institute Nofima.

Whole genome sequencing of *L. monocytogenes* isolates

Genome sequences of *L. monocytogenes* isolates from dairy farms (79), rural and urban environments (115) and slugs (24), as well as isolates from

patients with listeriosis (111) were compared in the study. All sequences from patients included in the study, were downloaded from public databases.

The analyses used for detection of *L. monocytogenes* in dairy farm samples had some minor differences compared to the methods used by Nofima. The dairy farm samples were analyzed according to modified NMKL No 136, 5th ed. 2010 and plated on ALOA, while those from rural and urban environments and from slugs were analyzed according to modified ISO 11290-1 and plated on RAPID`L.mono. Both modified methods make use of only one selective media, not two different as described in the two original methods. The two methods are very similar and were not expected to add any bias to the results. DNA isolated from 79 dairy farm isolates were subjected to paired end sequencing (2 x 150) on a NovaSeq 6000 S4 flow cell (Illumina). Only one colony were selected from each positive ALOA plate (regardless of the number of typical *L. monocytogenes* colonies appearing on the plate), potentially leaving out isolates with other sequence types present in the samples. The DNA samples isolated by Nofima, were sequenced on a MiSeq instrument (Illumina), using 2 x 300 bp paired end sequencing. A potential advantage for the Nofima sequences is that longer read lengths cover longer repetitive elements, that can provide larger contigs.

wgMLST analysis

The genome sequences of the dairy farm isolates were prior to submission to NCBI GenBank trimmed, refined and assembled using SPAdes v3.14.1 incorporated in a pipeline called Shovill (https://github.com/tseemann/shovill). To avoid bias related to using different bioinformatic tools, the dairy farm genome sequences used for phylogenetic analyses were assembled using the same method as used for the genomes sequenced at Nofima, with *de novo* genome assembly by SPAdes v3.10.0 or v3.13.0 [113]. The wgMLST analyses were based on blasting 4,797 coding loci in BioNumerics 7.6

(https://www.bionumerics.com/news/listeria-monocytogenes-whole-genome-sequence-typing). In general, wgMLST analysis methods is by some researchers considered more biological relevant than whole genome single nucleotide polymorphism (wgSNP) analysis [108]. wgMLST is based on allelic variation and considers deletions, insertions and recombinations in multiple positions as single evolutionary events. Single nucleotide polymorphism (SNP) analysis focuses only on point mutations and is dependent on using a good reference genome. If a gene is not present in the chosen reference, the SNP variant calling will not account for that gene. wgMLST is based on the pangenome, therefore it considers a larger percentage of the genome in its analyses [108]. The wgMLST pangenome can continuously be improved by adding new alleles as they are detected. The disadvantages of performing wgMLST is that it requires continuous allele curation for thousands of loci, and you need automated tools to handle all the data.

The term "strain" is in this thesis defined as descendants of a single isolate, while clones are genetically related isolates, presumably derived from a common origin [114]. For the purpose of investigating or initiating investigations of suspected *L. monocytogenes* outbreaks, regulators often look for clusters of isolates that have less than seven to 10 cgMLST allelic differences [102, 105, 115] or 20 SNPs [116, 117] in SNP analyses. Many of the isolates in our study clustered at a similar threshold, however, determining whether isolates match only based on cgMLST/wgMLST allelic differences or SNPs can be misleading. The genomes of food-, environment-, and clinical isolates can change by evolutionary forces, and the thresholds are controversial [118-120].

Paper III

The purpose of this paper was to explore growth properties and the pathogenic potential of the four *eae* positive STEC isolates isolated from Norwegian dairy farms in Paper I. Raw milk were inoculated with the isolates and incubated at different temperatures to illustrate effects of temperature abuse on STEC. The volume of milk stored at abused temperature will impact the time needed for reaching a specific temperature. The sample volume used in this study was 40 mL and do not necessarily reflect the normal volume of milk stored in consumers' homes. After one hour in room temperature (20°C), 40 mL milk initially holding 4°C reached a temperature of 18°C. It reached 20°C after 1.5 hours, and then it took three hours before it was back at 4°C. Larger volumes of milk will have delayed fluctuation of temperature, affecting the microbial growth.

To best mimic a real-life situation, we used raw milk with its natural microbial flora for the storage experiment. Raw milk is a more variable substrate than pasteurized milk, as the natural competitive microflora varies between batches. We aimed to use the same batch of BTM collected for the entire storage experiment. However, due to addition of extra experiments at 6°C we got short of milk from the same batch. Therefore, a new BTM batch was collected from the same farm and used for the last replicate for some samples. The results acquired from the second BTM batch were comparable to those from the first milk batch, observed by using both batches for each strain in the last replicate. The BTM were aliquoted into 50 mL Falcon tubes and stored at – 20°C until use. However, the three biological replicates were performed over a span of 6 months and the quality of the raw milk in the freezer may have deteriorated during this time. The milk separated in two layers during freezing, and the last replicate experiment required somewhat more mixing to get the milk homogenic.

To ensure comparable conditions during the experiment, the milk was inoculated with bacterial cultures of similar OD (OD_{600} 0.3) which gave a start concentration of STEC of approximately $3x10^3$ colony-forming units (CFU)/mL. In comparison, Giacometti *et al*. 2012 tested milk with a pathogen level of 50-100 CFU/mL, a concentration that was assumed to be a good simulation for raw milk contamination [23]. The high inoculum level in our study is not comparable to natural contamination levels but was used to facilitate for counting colonies on agar plates. It has previously been shown that the inoculum level affects the growth of streptomycin-resistant *E. coli* O157:H7 in raw milk supplemented with streptomycin as this strain grew faster when the inoculum concentration was low [121]. Instead of using plating techniques to examine the level of STEC in raw milk, quantitative real time PCR (qRT-PCR) may be used to study the pathogen concentration. qRT-PCR allows detection of *E. coli* present at lower levels, and the experimental design could thus be closer to the real-life situation.

As raw milk is a good microbial growth medium and has a relatively short shelf life, we decided to examine the levels of STEC in the inoculated milk samples after 24, 48 and 72 hours of storage. In reality, storage advice given by the manufacturers and authorities to consumers are often not followed, and it would be beneficial to store for a longer time to get a better overview how the pathogens behave in milk also in the long term. Leclair *et al*. (2019) investigated how time and temperature affected growth of *E. coli* O157:H7 in raw milk, and they monitored pH to see how souring of raw milk affected behavior of the pathogen [121]. During their study, the pH in the milk gradually decreased during storage for 10 days at 22°C, and a more rapid pH decrease was observed at low pathogen inoculum levels. The concentration of *E. coli* O157:H7 started to decrease from day four [121]. The decrease may have been a result of acidification of the growth substrate but other studies have shown that *E. coli* O157:H7 has caused outbreaks through acidic foods

like juice [122]. Giacometti *et al*. 2012 registered the pH in raw milk inoculated with *L. monocytogenes*, *E. coli* O157:H7, *Salmonella* Typhimurium and *C. jejuni*. The study indicated an increase in competitive microflora and a decreasing pH after 96 hours of storage in 4°C and also in samples incubated at variable temperatures ranging from 7 to 30°C [23]. It would have been informative to measure pH in the samples during our experiments to ensure more similar conditions in the replicates, and to be able to assess how a decreased pH affect the growth of STEC. However, *E. coli* O157:H7 has previously been shown to be a good competitor and to have good acid tolerance [23, 123]. We also had a shorter incubation time than previous studies which may have reduced the effect of pH decrease [121].

During the storage experiment, the raw milk was inoculated with STEC strains and incubated at different temperatures before plating on CHROMagar STEC for enumeration. When plating raw milk to CHROMagar STEC plates, we sometimes experienced a "swarming-like" growth, as if one colony got smeared out and caused growth of many small colonies. If plate was still readable, these smeared colonies were counted as one colony. Thorough spreading of sample material until the agar was dry prevented this "swarming" growth.

Initially, the samples were stored at 4°C, 8 and 20°C but when we saw the results, we decided to store samples at 6°C, as well as check growth of the four STEC isolates in Lysogeny broth (LB) at 8 and 20°C. The latter conditions were chosen to observe the effect of competitive bacterial flora or other antimicrobial activity by milk constituents in the raw milk samples. To mimic a real-life situation where milk is kept out of the refrigerator during meals, the milk was stored at 4°C except for 1.5 hours a day at 20°C. This setup was based on subjective assumptions on consumer handling practices.

Four *eae*-positive STEC isolates were whole genome sequenced using MinION nanopore long read sequencing. Long read sequencing were chosen as short-read sequencing methods can miss some segments of the genome and assembly of phages containing repetitive elements may be more difficult when short read sequencing is applied. *De novo* whole genome assembly resulted in closed genomes of single chromosomes ranging in size from 5.2 Mb to 5.5 Mb, in addition to a single plasmid for each isolate in the size range 55-80 Kb.

STEC isolate S2, S3 and S4 were collected from the same farm. S3 and S4 were collected at the same day, S3 from a milk filter sample and S4 from cattle feces. They show high genetic similarities, and it is likely to think that these rise from the same ancestor. The S2 isolate was collected approximately 5 months earlier at the same farm and it is genetically distinct from S3 and S4. S1 is genetic very similar to S3 and S4, but from a different farm and collected seven months prior to collection of S3 and S4. The two farms are located in the same area, within a radius of 10 km. STECs have been shown to persist in cattle farm environments over time [124], and the persistence of STEC strains may contribute to increased contamination- and recontamination pressure on the raw milk produced at a farm.

Shiga toxin-converting phages (Stx phages) occur as prophages in STEC cells. In the lysogenic state, most phage genes are not expressed, *stx* genes included. Stress conditions can induce the prophage multiply, which leads to host cell lysis and release of virions together with Stx. STECs can also become infected with other bacteriophages that can lysate the bacteria [125] and make it difficult to grow in overnight culture and to get correct results from enumerating colonies by plate counting. The genomes of the STEC strains included in the study were not examined for the presence of non-Stx bacteriophages.

The Stx detection kit RIDASCREEN® Verotoxin (R-biopharm, Darmstadt, Germany), used in this study, is shown to detect all known variants of Stx1 and Stx2 [126]. Mitomycin C (MMC) is known as a highly potent inducer of Stx production [127, 128] and was used to induce cultures at OD 0.5. Toxin production was examined 150 minutes after induction as Stx has been shown to be clearly detectable by SDS-PAGE 180 minutes post induction with MMC [129]. To read the results (OD_{450}) within the correct range, the culture supernatants were diluted 1:20 prior to analysis of Stx production.

9 General discussion

The overall aim of this thesis was to address the risk for zoonotic infections associated with consumption of raw milk from Norwegian farms. First the thesis investigates the prevalence of *L. monocytogenes*, *Campylobacter* spp., and STEC in raw milk from farms with different operating systems. Then, to get an insight into transmission pathways and persistence of *L. monocytogenes* in different habitats, isolates from farm environments and raw milk were compared phylogenetically with isolates from both other environmental habitats and listeriosis patients. Finally, the risk of attracting zoonotic disease related to consumers behavior, was addressed by testing the ability of STEC to grow in raw milk stored at abused temperature.

9.1 Occurrence of zoonotic pathogens in raw milk samples

For many years, raw milk has been a known transmission route for human pathogens. Today, it is primarily *Campylobacter jejuni*, STEC, *L. monocytogenes* and *Salmonella* that are discussed in relation to the food safety challenge they represent [89, 130-132]. In the EU, TBEV is also considered a main hazard, as it is endemic in many parts of Europe and detected in raw milk [132]. *Brucella melitensis* and *Mycobacterium bovis* are less common in Europe as control programs have successfully reduced their distribution, but they are still linked to raw milk outbreaks [132].
In this project, we decided to focus on STEC, *Campylobacter* spp. and *L. monocytogenes*. In 2006 VKM performed a risk assessment on raw milk consumption and concluded "with the current epidemiological situation in Norway, the risk for transmission of *E. coli* O157:H7 and other EHEC, *C. jejuni* and *L. monocytogenes* to humans by consumption of raw milk and cream should be considered high" [17]. The NVI has an ongoing surveillance program for *Salmonella* and *Brucella* [57]. Brucella can be shred in milk, but

Norwegian cattle are officially free from this pathogenic agent and the disease has never been detected in sheep or goats in our country [57]. The prevalence of *Salmonella* in Norwegian cattle is low [101], and the number of human cases have decreased the last 10 years in Norway. The low number of remaining cases are often linked to imported foods [57]. As Norway is officially free from bovine tuberculosis [57], this bacterium together with *Salmonella* and *Brucella* were excluded from our project.

Studies from the 80s and the 90s, performed in USA and Canada, reported a prevalence of *C. jejuni* between 0.4 to 9.2% [51, 91, 133-136] in BTM samples, while *L. monocytogenes* had a prevalence between 1.6 to 5.4% [91, 133, 135-137] and STEC between 0.9 to 3.8% [91, 136]. After year 2000, *C. jejuni* was isolated from 2% [93] and 0.34% [99], *L. monocytogenes* from between 0.68 to 6.5% [93, 96, 99, 138], and STEC from 2.4% [93] and 0.8% [139] of BTM samples collected in Sweden and USA. In our study, we did not detect *L. monocytogenes* in BTM, while *Campylobacter* spp. were detected in 3% of the BTM samples and STEC in 1% (only one positive sample). The acquired prevalence of pathogens in BTM can be influenced by several factors, like variations in sampling procedures, different methods for analysis, geographical location, season and climate changes, herd size and dairy farm management practices [136].

9.2 Occurrence of zoonotic pathogens in dairy farms with different management systems

Different herd-management strategies, regulating animal density, sanitation of bedding, feed production, drug treatment of cattle and cattle trade, can influence microbial populations in the farms. During this project, we have examined how loose versus tie-stall housing affect the occurrence of three selected pathogens. Several studies have been done to investigate possible connections between farm operational system and somatic cell count (SCC)

and total bacterial count (TBC) in bulk tank milk [140-145]. Previous studies have reported increased somatic cell count and total bacterial count in bulk tank milk after implementation of AMS. These increases often persist several years after the transition [140, 141, 146-148]. The reduction in milk quality may relate to other factors than directly to the implementing of a new milking system, as some studies show similar SCC and TBC between farms using either AMS or CMS [149, 150]. Factors like irregularity of milking intervals, milking failure and increased milking frequency can influence the level of bacteria and somatic cells in the raw milk. Frequent milking reduces the chance for microorganisms to colonize the quarters but, on the other hand, teat sphincters that are more often open allows for microbial invasion of the quarters [151]. To our knowledge, not much research has been done to investigate how transition from tie-stall housing to loose housing influence the level of zoonotic microorganisms in the milk as other milk quality parameters have been in focus. In the thesis, we observed a higher occurrence of pathogens in loose housed herds compared to tie-stall herds in the following combinations of agents and samples: *L. monocytogenes* in feces and silage and *Campylobacter* spp. in feces and teat swab samples. The presence of the gene encoding intimin, a protein important for EHEC during infection, was also more prevalent in BTM and milk filter in loose housed herds. However, stalling, and milking strategy is connected, and to conclude on the specific factor that cause the differences in pathogen occurrence is not straight forward. Loose housing herds often have a milking robot, a milking parlor or carousel, while tie-stall herds more often have pipeline milking directly in the stall. Insufficiently cleaned teats are considered the main reason for milk contamination [152], and both stalling conditions and milking process can affect the teat contamination level.

In loose housing the number of animals per area might be lower compared to tie-stall housed herds because the animal buildings used for loose housing are often larger. However, there is a higher possibility for direct contact

between animals, for example when they share the feeding and bedding area. Hence, teats can also get dirty with feces from other animals. AMS robots are automated by sensors and analyzers, and there is no visual control during the milking. In contrast to CMS where infected cows often are milked last to prevent pathogen transmission, the milking in AMS is random with steaming of milking lines between cows. The AMS have automated teat washing, that do not account for the individual cows hygiene status, and satisfactory teat washing is not always achieved [151], however studies indicate that the milking robot is sufficient to remove dirt and spores from the teats [153]. AMS and loose housing systems were introduced to improve animal welfare, reduce farmer workload, and increase profit [5, 154]. The new technologies provide a lot of advantages, but also some disadvantages that one must be aware of, and more research is needed to further improve the microbial contamination status of raw milk from AMS [151].

Milk quality is impacted by several environmental factors, including cow cleanliness [155-157]. The cleanliness status of cows depends on housing type, stall structure, bedding material, season, weather, manure management, air humidity and animal type [155, 156]. Poor farm hygiene expose the animals for more environmental pathogens [155], and poor udder hygiene increase the risk of mastitis and other cattle infections [155, 157, 158]. To explore the relationship between farm hygiene and milk quality we investigated the association between cow cleanliness and detection of the three pathogens in BTM, milk filter and feces, and for *L. monocytogenes* and *Campylobacter* spp. also in teat swab and teat milk. Only *Campylobacter* spp. detected in teat milk had a significant association to cow hygiene score. However, larger studies may reveal additional positive associations.

9.3 Distribution of *L. monocytogenes* strains across natural, agricultural, and urban environments

In paper II we investigated the genetic relationship between *L. monocytogenes* collected from natural, agricultural, and urban environments in Norway, and compared the findings to isolates found in Norwegian clinical cases. *L. monocytogenes* is known to be widely distributed in natural environments [159], but less is known about its persistence in particular habitats and the coherence between isolates from different sources. The emergence of WGS has provided new and better opportunities to study genetic diversity and contamination routes for pathogenic microbes and has become an important tool in outbreak analysis [102]. Multi-locus sequence typing (MLST) was in the current study used to identify *L. monocytogenes* clonal groups whereas wgMLST was used to study the evolutionary linkage between isolates. The isolates were grouped into STs, defined by distinct combinations of alleles for seven housekeeping genes [160], and into CCs that were defined as groups of ST profiles sharing at least six of the seven genes with one or more members of the group [161]. The most prevalent CCs in the study (rural/urban, farm, slug) were the linage II clones CC91, CC11 and CC37. Isolates belonging to lineage I occurred less frequently in this study, with CC1, CC4 and CC6 being the most commonly detected.

Interestingly, Chenal Francisque *et al*. 2011 [162] found CC2, CC9 and CC121 to be most prevalent CCs in environmental or vegetation samples collected worldwide, while CC1 and CC2 were most prevalent in samples from food and animals collected worldwide. Bergholz *et al*. 2018 [163] state that the historically common CC1 and CC2 are less frequent today, and other CCs like CC5, CC6, CC9 and CC121 are emerging. In our study none of the isolates were of CC2 or CC5, and only a single isolate belonging to CC9 and CC121 were detected. However, there are similarities between our isolates and the global clonal composition. Isolates belonging to CC1 (ST1) were collected from nine

samples and was the most common lineage I CC detected in our study and CC6 was also frequently isolated. Accumulation of some specific clones in multiple locations is a field of interest, raising questions regarding connections between clonal dispersal and increased travel, trade and migration of animals and humans.

The literature indicates that dairy farm environments contain a diversity of *L. monocytogenes* strains [164]. We examined the genetic linkage between clones found in milk filters and farm environments to study sources of BTM contamination. Some of the isolates found in milk filters were closely related to clones found in feed, feces and teat swabs, indicating that the bacteria can be transferred from the environment into the milking system. Listeria mastitis has been discussed as a potential contamination route, but it is probably more likely that the bacteria are introduced to the milking system through the cow udder surface during milking, as *Listeria* mastitis is quite rare [37]. Many studies have isolated *L. monocytogenes* from several environmental sites within a dairy farm [38, 165], suggesting a high contamination pressure from for example feed and drinking water and other animals shedding the bacterium in feces. However, some of the clones we found in milk filters were not detected in the environment at the same farm. This might be due to great strain diversity within the species and shortcomings in the used methods. A weakness in the study design is that only one colony from each dairy farm sample was selected for WGS, and if samples contained isolates belonging to multiple STs, these may thereby have escaped detection, even if present. As *L. monocytogenes* is a known biofilm producer [35], another possible reason for milk contamination can be biofilm formation on surfaces in the milking system, causing specific clones to persist over time without being continuously introduced from outside sources. We did not detect the same clone in milk filters over time at any of the farms

included in this study, but one farm had positive milk filter at four different occasions, with different STs each time.

We sampled different locations repeatedly to study strain dynamics over time. The suspicion that specific clones persist within a certain environment was reinforced as 12 isolate clusters were present at a dairy farm at more than one occasion, with a time span of 2 to 10 months. With 0 to 11 wgMLST allelic differences, these isolates show close genetic linkage, increasing the likelihood that they have a common origin. *L. monocytogenes* has been shown to persist in milking systems [166] and food producing environments [167, 168], and studies indicate that some specific clones have a better ability to persistent in food production environments than others [169, 170]. We found ST4, ST8, ST18, ST2761, ST37, ST91, ST177, ST226, ST394, ST412, and ST451 to be persistent in the dairy farms. ST8 (CC8) is also frequently detected in food production premises and has shown ability to persist in such environments [167, 171]. This CC has also been identified as the cause of clinical listeriosis cases in humans [172, 173].

The same *L. monocytogenes* clone was detected on more than one farm in the same geographical area on three occasions, with allelic differences ranging from 11 to 19 wgMLST alleles. This is within the threshold often suggested for defining an outbreak cluster [102, 105, 115]. The farms were not in immediate proximity to each other, leaving the potential contamination route an open question. *L. monocytogenes* is ubiquitous in soil and is often believed to enter farm environments through silage contaminated during harvest [36]. The cases where the same clone was detected on different farms involved at least one feed sample, indicating feed to be a possible common source of contamination. However, in Norway, strict hygienic measures are often practiced on farms, and many farmers avoid purchasing livestock or feed from other farms to avoid the spread of infectious agents. The farms included

in this study all produced their own silage the year of sampling, leaving this to be no obvious reason for the presence of related clones at different farms. It is unknown if the different croplands used for harvesting silage material are located near each other, but often these fields are placed near the farm building. Some cattle herds have common pasture to other herds or other husbandry animals like sheep, this was not registered in this study. A cluster of eight ST451 isolates from five farms were detected, and these farms were not located in the same geographical area. Six clusters comprised isolates from both dairy farms and from urban/rural environments. Altogether, this study indicated that different and distant locations can host the same *L. monocytogenes* clone.

ST9 (CC9) is frequently found in the meat processing industry [109, 174] in Norway. A Spanish study [175] that sampled meat processing plants and meat also found ST9 to be the predominant ST isolated (33% of the isolates), followed by ST121 (CC121) (16%). In France, CC9 and CC121 were also the most prevalent CCs in pork production sector [176]. Maury *et al*. 2019 [177] link CC1 to dairy products and CC9 and CC121 to meat products. CC9 and CC121 were not detected in any of our dairy farm samples, nor were the food and infection-associated clones CC1 or CC2 [161]. This might indicate that the food associated clones do not enter the food production chain at farm level, but at a later stage. As we only sampled dairy farms, samples from specific meat production herds can hold other isolates than the ones we found. However, the most prevalent dairy breed in Norway, the Norwegian Red, is also used for meat production, leaving meat-associated clones also relevant at dairy farms, and *vise versa*. As previously mentioned, we only sequenced one isolate from each sample, leaving potential additional clones undetected. Kim *et al.* 2018 [178] show that the predominating CCs isolated from BTM in the US is CC7 and CC37 and this study also indicate that a significant proportion of isolates detected in BTM and milk filters belong to the same CCs as those

that frequently cause human disease. In our study, the predominating CC from milk filters and dairy farm environments was CC11 (one ST11, 14 ST451 and one ST2760), which comprised 20 % of the dairy farm isolates. It was followed by CC91 (15%), and CC37 (11%). These percentages are similar to those found in a Latvian study [179] where CC37 (30%), CC11 (20%) and CC18 (17%) were most frequently isolated from dairy farm animal- and environment samples.

9.4 *L. monocytogenes* typing and nomenclature

L. monocytogenes surveillance and typing have used a lot of different methods over the years [102]. Species identification has previously been based on phenotypic properties, serotyping and PFGE. Later implementation of sequence-based methods like multi-locus sequence typing (MLST) has improved bacterial typing by providing standardized nomenclature [102]. Based on seven housekeeping genes, MLST could group *L. monocytogenes* isolates into STs and CCs [104]. However, PFGE do not reflect evolutionary relationships, and both PFGE and MLST is suspected do have insufficient discriminative power for outbreak surveillance [102]. The Norwegian Institution of public health (FHI) is monitoring infectious diseases in Norway. It is obligatory to report listeriosis cases to the Norwegian Surveillance System for Communicable Diseases (MSIS) (FOR-2003-06-20-740), and FHI is currently typing all isolates using MLST [104] together with cgMLST [105]. These methods identify STs and CT, respectively. Typing by cgMLST is based on analysis of over thousand genes, 1701 for the scheme used by FHI [105], and is much more informative than MLST, and thereby makes it easier to avoid false clustering of isolates. However, in Paper II we compare *L. monocytogenes* isolates collected at the dairy farms with isolates collected in other parts of Norway, both human clinical- and environmental isolates, using wgMLST. The analyses revealed that clusters of isolates with no likely association were indistinguishable using cgMLST analysis and even wgMLST

and SNP. This indicates the need for further improvements of the surveillance systems used today, and to not rely only on the DNA-analyses. To gain knowledge on the diversity and similarities between environmental-, food-, and clinical bacterial strains, it is important to publish acquired genome sequences, allowing the research community to compare sequences from different sources. To face future challenges of multi country pathogen outbreaks and pandemics, it is crucial to implement international surveillance and pathogen strain subtyping strategies that allow rapid identification of true outbreak clusters, as well as to have a universally shared nomenclature that improves global collaboration. Historically, listeriosis has mostly been organized at national levels, which limits the chance of tracing cross country transmission events [180]. As food trade and human travel increases, pathogen circulation and international outbreaks will probably follow, highlighting the need for rapid identification of epidemiologically linked isolates.

9.5 The risks of raw milk storage

Lactic acid bacteria (LAB) are naturally occurring in milk and can have some antimicrobial effect on pathogens [181], but long time storage of raw milk is strongly discouraged. Most pathogens will not proliferate under correct storage conditions, except for psychotropic bacteria like *Listeria* spp., that can grow in refrigerator temperatures [18, 23]. Other pathogens, like *Campylobacter* spp. [182] and STEC, have a low infectious dose and *E. coli* O157:H7 is speculated to be able to cause disease from only 10 to 100 cells [121]. Thus, raw milk can be a health risk to consumers regardless of bacterial multiplication. In a study on how field handling conditions of raw milk affected the behavior of pathogens, two different temperature settings were examined [23]. The first to simulate optimal storage conditions at 4°C, and the second to simulate the worst conditions registered for raw milk being sold from vending machines (data obtained from a preliminary study). For

the second group, the samples were stored to illustrate the process of transportation to a vending machine (7.0°C±0.5 for 5 h), vending machine storage (11°C±0.5 for 22.5 h), transportation home (30°C±0.5 for 30 min) and home storage (12°C±0.5 for 68 h) [23]. *Campylobacter* spp. decreased at both temperature settings. *E. coli* O157:H7 remained unchanged at 4°C but showed growth at the higher temperatures.

Consumer food handling practices has proven to be unsatisfactory when it comes to time and temperature [23, 183], increasing the risk of attracting foodborne disease. Even though boiling is recommended before UPM consumption, studies show that this is often not done [23], probably because it contradict what is appealing with that type of milk. In our study, we observed that the four STEC isolates from paper I were able to multiply in raw milk stored at 8°C (Paper III), and indications of growth were also observed at 6°C. However, the STEC level decreased at 4°C indicating that storage of raw milk at temperatures ≤ 4°C is crucial for reducing the risk of getting sick by consumption of raw milk.

Inoculated raw milk samples kept at 4°C, except for exposure to room temperature for 1.5 hours per day, showed that one of the isolates were able to multiply in raw milk under these conditions. This indicate that storage at room temperature for relatively short periods of time may cause propagation of STEC in raw milk, even though it is kept at optimal refrigerator temperature most of the time. This result highlights the need for an intact cooling chain to keep raw milk as safe as possible. Due to the low infectious dose of STEC, even a small increase in concentration may increase the risk of food-borne disease significantly.

Milk can harbor a variety of pathogens and is mainly contaminated after contact with fecal material during milking, in the milking system, via milking personnel or from farm environment, often by dirty teats [151]. Measures to

reduce pathogen carriage and extensive hygienic practices in the farm, like barn hygiene, sanitation of milking equipment and controls of the milking process [151], can reduce raw milk contamination. It is however important to note that such measures will never eliminate the presence of pathogens at dairy farms and the risks associated with consumption of raw milk completely.

10 Conclusions

L. monocytogenes, *Campylobacter* spp., and STEC are zoonotic bacteria that can cause severe milk borne disease. They are commonly found in Norwegian dairy farms, and it is difficult to avoid transmission of these disease agents to raw milk. It is therefore important to maintain the cold chain throughout transportation and storage of raw milk to prevent multiplication of bacteria. With the low infectious dose of *Campylobacter* spp. and EHEC, only small quantities of contaminated food are needed to cause food borne illness. Continuously changing agricultural technologies present new food safety challenges, and the modern loose housing systems are associated with increased levels of pathogens in the farms. *L. monocytogenes* can persist in dairy farm environments for months and can contaminate milk in cross-contamination events. Even though good on-farm hygiene can reduce the risk of milk being contaminated by pathogens, it does not eliminate the risk. Consumption of Norwegian raw milk presents a risk for contracting infectious diseases, especially in susceptible groups like young children and elderly, highlighting the importance of preventative measures like pasteurization to ensure safe drinking milk.

11 Future perspectives

As the raw food movement is growing, food safety related challenges are likely to increase. These challenges call for action, and new knowledge is therefore needed to facilitate informed decision making regarding raw milk politics and to ensure safe drinking milk. Some individuals and communities are skeptical to authority warnings, highlighting the importance of clear communication of the risks associated with raw milk consumption. Despite new milking practices and more modern cleaning and sanitizing procedures in the farms, the food safety risk associated with raw milk consumption is still relevant.

A wider understanding of how pathogenic clones emerge and are distributed in the environment is needed to identify possible contamination routes both into the farm areas and ultimately to the raw milk. Furthermore, to develop better regimes to reduce bacterial contamination of raw milk, we need more knowledge on bacterial persistence in farm environments and of where in the milking systems pathogens settle. This is also of outmost importance for making production of milk and milk products more sustainable as bacterial contamination is a universal problem that leads to waste of food due to suboptimal quality and limited shelf life, also in pasteurized products. Ultra-high temperature (UHT) pasteurized milk is adapted for long term storage, but this processing of milk affects the taste and is not commonly used in the Nordic countries.

We need more information on the prevalence of food borne disease cases caused by UPM. The number of foodborne disease cases associated with UPM is probably underreported in Norway as outbreaks are small and infections are usually healed within a relatively short time in healthy individuals. However, the use of raw milk in Norway is still limited, and a more extensive

use we will probably result in more disease cases, especially in susceptible groups. Communication of research results and knowledge to the public is, therefore, important to promote safe handling of UPM products. Consumers need more knowledge regarding storage time and temperature conditions suitable to prevent growth and survival of different pathogens. Persons who have a preference for drinking UPM or giving it to their children should also be made aware that the risks associated with consumption of UPM is especially applicable to young children, elderly and immunocompromised individuals.

In the increasingly globalized world, improved international research cooperation to study similarities and dissimilarities between foodborne microorganisms are important to develop strategies to avoid contamination of food. There is little doubt that climate changes will affect the distribution of pathogens in the environment in the future. As we expect warmer and more humid weather in Norway, receiving knowledge from countries which already have experience of producing milk and milk products under such conditions would help us to meet food safety and quality related challenges in the future.

There is very limited knowledge on why some *L. monocytogenes* clones are more prevalent in certain environments, calling for a more research on what underlaying properties that determines their preference for specific environmental habitats and how they enter the food chain. By using WGS we can investigate how bacterial clones arise, spread, and persist in the environment, animal-, and human-populations, as well as perform improved foodborne disease outbreak analyses. The enormous volumes of data generated, at an increasing pace, demands good and intuitive bioinformatic tools to interoperate findings, and databases housing genome information available all over the world. A strong knowledge base is needed when

developing public health strategies that will help us to meet present and future food safety related challenges.

12 References

1. Evershed RP, Payne S, Sherratt AG, Copley MS, Coolidge J, Urem-Kotsu D, et al. Earliest date for milk use in the Near East and southeastern Europe linked to cattle herding. Nature. 2008;455(7212):528-31; doi: 10.1038/nature07180.
2. TINE. Årsrapport 2021. https://www.mynewsdesk.com/no/tine-sa/documents/tine-aarsrapport-2021-dot-pdf-419567; 2022. Accessed October 31, 2022.
3. Bjørkhaug H. Sustainable agriculture in the Norwegian farmers' context: Exploring farming habitus and practice on the Norwegian agricultural field. Int J Environ Cult Econ Soc Sustain. 2006;4(2):123-31; doi: 10.18848/1832-2077/CGP/v02i04/54241.
4. Almås R, Brobakk J. Norwegian dairy industry: A case of super-regulated co-operativism. In: Rethinking Agricultural Policy Regimes: Food Security, Climate Change and the Future Resilience of Global Agriculture. Emerald Group Publishing Limited; 2012. p. 169-89.
5. Vik J, Stræte EP, Hansen BG, Nærland T. The political robot–The structural consequences of automated milking systems (AMS) in Norway. NJAS-Wagen J Life Sci. 2019;90:100305; doi: 10.1016/j.njas.2019.100305.
6. Jacobs JA, Siegford JM. Invited review: The impact of automatic milking systems on dairy cow management, behavior, health, and welfare. J Dairy Sci. 2012;95(5):2227-47; doi: 10.3168/jds.2011-4943.
7. Cogato A, Brščić M, Guo H, Marinello F, Pezzuolo A. Challenges and Tendencies of Automatic Milking Systems (AMS): A 20-Years Systematic Review of Literature and Patents. Animals (Basel). 2021;11(2):356; doi: 10.3390/ani11020356.
8. Smistad M, Kaspersen H, Franklin-Alming F, Wolff C, Sølverød L, Porcellato D, et al. *Streptococcus dysgalactiae* ssp. *dysgalactiae* in Norwegian bovine dairy herds: Risk factors, sources, and genomic diversity. J Dairy Sci. 2022;105(4):3574-87.
9. Statistics Norway. Statistics for Agriculture, Forestry, Hunting and Fishing. https://www.ssb.no/en/jord-skog-jakt-og-fiskeri/statistikker/jordhus; 2021. Accessed October 31, 2022.
10. Heringstad B. Genetic analysis of fertility-related diseases and disorders in Norwegian Red cows. J Dairy Sci. 2010;93(6):2751-6; doi: 10.3168/jds.2009-2879.
11. Holtsmark M, Heringstad B, Madsen P, Ødegård J. Genetic relationship between culling, milk production, fertility, and health traits in Norwegian Red cows. J Dairy Sci. 2008;91(10):4006-12; doi: 10.3168/jds.2007-0816.

12. Garmo RT, Waage S, Sviland S, Henriksen BIF, Østerås O, Reksen O. Reproductive Performance, Udder Health, and Antibiotic Resistance in Mastitis Bacteria isolated from Norwegian Red cows in Conventional and Organic Farming. Acta Vet Scand. 2010;52(1):11; doi: 10.1186/1751-0147-52-11.
13. Quigley L, O'Sullivan O, Stanton C, Beresford TP, Ross RP, Fitzgerald GF, et al. The complex microbiota of raw milk. FEMS Microbiol Rev. 2013;37(5):664-98; doi: 10.1111/1574-6976.12030.
14. Fusco V, Chieffi D, Fanelli F, Logrieco AF, Cho GS, Kabisch J, et al. Microbial quality and safety of milk and milk products in the 21st century. Compr Rev Food Sci Food Saf. 2020;19(4):2013-49; doi: 10.1111/1541-4337.12568.
15. O'connell A, Ruegg P, Jordan K, O'brien B, Gleeson D. The effect of storage temperature and duration on the microbial quality of bulk tank milk. J Dairy Sci. 2016;99(5):3367-74; doi: 10.3168/jds.2015-10495.
16. Hanson M, Wendorff W, Houck K. Effect of heat treatment of milk on activation of *Bacillus* spores. J Food Prot. 2005;68(7):1484-6; doi: 10.4315/0362-028X-68.7.1484.
17. Wasteson Y, Blom H, Fossum K, Høiby EA, Narvhus J, Håvarstein LS, et al. A qualitative assessment of the risks of transmission of microorganisms to humans resulting from the consumption of raw milk and raw cream in Norway. Panel for Biological Hazards, Norwegian Scientific Committee for Food Safety; 2006.
18. Castro H, Ruusunen M, Lindström M. Occurrence and growth of *Listeria monocytogene*s in packaged raw milk. Int J Food Microbiol. 2017;261:1-10; doi: 10.1016/j.ijfoodmicro.2017.08.017.
19. Langer AJ, Ayers T, Grass J, Lynch M, Angulo FJ, Mahon BE. Nonpasteurized dairy products, disease outbreaks, and state laws-United States, 1993-2006. Emerg Infect Dis. 2012;18(3):385-91; doi: 10.3201/eid1803.111370.
20. Lucey JA. Raw Milk Consumption: Risks and Benefits. Nutr Today. 2015;50(4):189-93; doi: 10.1097/NT.0000000000000108.
21. Kenyon J, Inns T, Aird H, Swift C, Astbury J, Forester E, et al. *Campylobacter* outbreak associated with raw drinking milk, North West England, 2016. Epidemiol Infect. 2020;148:e13; doi: 10.1017/s0950268820000096.
22. Mørk T, Bergsjø B, Sviland S, Kvitle B. Humanpatogene bakterier i tankmelk fra ku og geit. Norwegian Veterinary Institute, Oslo; 2003.
23. Giacometti F, Serraino A, Finazzi G, Daminelli P, Losio MN, Tamba M, et al. Field handling conditions of raw milk sold in vending machines: experimental evaluation of the behaviour of *Listeria monocytogenes*, *Escherichia coli* O157: H7, *Salmonella Typhimurium* and *Campylobacter jejuni*. Ital J Anim Sci. 2012;11(1):e24; doi: 10.4081/ijas.2012.e24.

24. Jørgensen HJ, Mørk T, Rørvik LM. The occurrence of *Staphylococcus aureus* on a farm with small-scale production of raw milk cheese. J Dairy Sci. 2005;88(11):3810-7; doi: 10.3168/jds.s0022-0302(05)73066-6.
25. Norwegian Food Safety Authority. Endring i regler for omsetning av rå melk og rå fløte til konsum. https://www.mattilsynet.no/mat_og_vann/produksjon_av_mat/melk_og_meieriprodukter/endring_i_regler_for_omsetning_av_raa_melk_og_raa_flote_til_konsum.32893; 2018. Accessed March 14, 2022.
26. Bryan FL. Epidemiology of milk-borne diseases. J Food Prot. 1983;46(7):637-49; doi: 10.4315/0362-028X-46.7.637.
27. European Food Safety Authority and European Centre for Disease Prevention and Control. The European Union One Health 2020 Zoonoses Report. EFSA J. 2021;19(12):e06971; doi: 10.2903/j.efsa.2021.6971.
28. Costard S, Espejo L, Groenendaal H, Zagmutt FJ. Outbreak-related disease burden associated with consumption of unpasteurized cow's milk and cheese, United States, 2009-2014. Emerg Infect Dis. 2017;23(6):957-64; doi: 10.3201/eid2306.151603.
29. Johnsen BO, Lingaas E, Torfoss D, Strøm EH, Nordøy I. A large outbreak of *Listeria monocytogenes* infection with short incubation period in a tertiary care hospital. J Infect. 2010;61(6):465-70; doi: 10.1016/j.jinf.2010.08.007.
30. Bergholz TM, den Bakker HC, Katz LS, Silk BJ, Jackson KA, Kucerova Z, et al. Determination of Evolutionary Relationships of Outbreak-Associated *Listeria monocytogenes* Strains of Serotypes 1/2a and 1/2b by Whole-Genome Sequencing. Appl Environ Microbiol. 2016;82(3):928-38; doi: 10.1128/AEM.02440-15.
31. Marshall KE, Nguyen T, Ablan M. Investigations of Possible Multistate Outbreaks of *Salmonella*, Shiga Toxin-producing *Escherichia coli*, and *Listeria monocytogenes* Infections - United States, 2016. MMWR Surveill Summ. 2020;69(6):1-14; doi: 10.15585/mmwr.ss6906a1
32. European Food Safety Authority and European Centre for Disease Prevention and Control. The European Union One Health 2018 Zoonoses Report. EFSA J. 2019;17(12):e05926-n/a; doi: 10.2903/j.efsa.2019.5926.
33. Castro H, Jaakkonen A, Hakkinen M, Korkeala H, Lindström M. Occurrence, Persistence, and Contamination Routes of *Listeria monocytogenes* Genotypes on Three Finnish Dairy Cattle Farms: a Longitudinal Study. Appl Environ Microbiol. 2018;84(4):e02000-17; doi: 10.1128/AEM.02000-17.
34. Lambertz ST, Ivarsson S, Lopez-Valladares G, Sidstedt M, Lindqvist R. Subtyping of *Listeria monocytogenes* isolates recovered from retail ready-to-eat foods, processing plants and listeriosis patients in

Sweden 2010. Int J Food Microbiol. 2013;166(1):186-92; doi: 10.1016/j.ijfoodmicro.2013.06.008.
35. Mazaheri T, Cervantes-Huamán BR, Bermúdez-Capdevila M, Ripolles-Avila C, Rodríguez-Jerez JJ. *Listeria monocytogenes* biofilms in the food industry: is the current hygiene program sufficient to combat the persistence of the pathogen? Microorganisms. 2021;9(1):181; doi: 10.3390/microorganisms9010181.
36. Driehuis F, Wilkinson J, Jiang Y, Ogunade I, Adesogan A. Silage review: Animal and human health risks from silage. J Dairy Sci. 2018;101(5):4093-110; doi: 10.3168/jds.2017-13836.
37. Jensen NE, Aarestrup FM, Jensen J, Wegener HC. *Listeria monocytogenes* in bovine mastitis. Possible implication for human health. Int J Food Microbiol. 1996;32(1-2):209-16; doi: 10.1016/0168-1605(96)01105-1.
38. Latorre AA, Van Kessel JA, Karns JS, Zurakowski MJ, Pradhan AK, Zadoks RN, et al. Molecular ecology of *Listeria monocytogenes*: evidence for a reservoir in milking equipment on a dairy farm. Appl Environ Microbiol. 2009;75(5):1315-23; doi: 10.1128/AEM.01826-08.
39. Altekruse SF, Stern NJ, Fields PI, DL S. *Campylobacter jejuni* - An Emerging Foodborne Pathogen. Emerging Infectious Diseases. 1999;5:23-35; doi: 10.3201/eid0501.990104.
40. Kaakoush NO, Castaño-Rodríguez N, Mitchell HM, Man SM. Global epidemiology of *Campylobacter* infection. Clin Microbiol Rev. 2015;28(3):687-720; doi: 10.1128/CMR.00006-15.
41. Ocejo M, Oporto B, Hurtado A. Occurrence of *Campylobacter jejuni* and *Campylobacter coli* in Cattle and Sheep in Northern Spain and Changes in Antimicrobial Resistance in Two Studies 10-years Apart. Pathogens. 2019;8(3):98; doi: 10.3390/pathogens8030098.
42. Coker AO, Isokpehi RD, Thomas BN, Amisu KO, Obi CL. Human campylobacteriosis in developing countries1. Emerging infectious diseases. 2002;8(3):237; doi: 10.3201/eid0803.010233.
43. Boysen L, Rosenquist H, Larsson J, Nielsen E, Sørensen G, Nordentoft S, et al. Source attribution of human campylobacteriosis in Denmark. Epidemiol Infect. 2014;142(8):1599-608; doi: 10.1017/S0950268813002719.
44. Taylor E, Herman K, Ailes E, Fitzgerald C, Yoder J, Mahon B, et al. Common source outbreaks of *Campylobacter* infection in the USA, 1997–2008. Epidemiol Infect. 2013;141(5):987-96; doi: 10.1017/S0950268812001744.
45. Lahti E, Rehn M, Ockborn G, Hansson I, Ågren J, Engvall EO, et al. Outbreak of campylobacteriosis following a dairy farm visit: confirmation by genotyping. Foodborne Pathog Dis. 2017;14(6):326-32; doi: 10.1089/fpd.2016.2257.

46. Davis KR, Dunn AC, Burnett C, McCullough L, Dimond M, Wagner J, et al. *Campylobacter jejuni* infections associated with raw milk consumption—Utah, 2014. Morb Mortal Wkly Rep. 2016;65(12):301-5; doi: 10.15585/mmwr.mm6512a1.
47. Longenberger AH, Palumbo AJ, Chu AK, Moll ME, Weltman A, Ostroff SM. *Campylobacter jejuni* infections associated with unpasteurized milk—multiple states, 2012. Clin Infect Dis. 2013;57(2):263-6; doi: 10.1093/cid/cit231.
48. Burakoff A, Brown K, Knutsen J, Hopewell C, Rowe S, Bennett C, et al. Outbreak of fluoroquinolone-resistant *Campylobacter jejuni* infections associated with raw milk consumption from a herdshare dairy—Colorado, 2016. Morb Mortal Wkly Rep. 2018;67(5):146; doi: 10.15585/mmwr.mm6705a2.
49. Heuvelink AE, van Heerwaarden C, Zwartkruis-Nahuis A, Tilburg JJHC, Bos MH, Heilmann FGC, et al. Two outbreaks of campylobacteriosis associated with the consumption of raw cows' milk. Int J Food Microbiol. 2009;134(1-2):70-4; doi: 10.1016/j.ijfoodmicro.2008.12.026.
50. Jaakkonen A, Kivistö R, Aarnio M, Kalekivi J, Hakkinen M. Persistent contamination of raw milk by *Campylobacter jejuni* ST-883. PLoS One. 2020;15(4):e0231810; doi: 10.1371/journal.pone.0231810.
51. Doyle MP, Roman DJ. Prevalence and survival of *Campylobacter jejuni* in unpasteurized milk. Appl Environ Microbiol. 1982;44(5):1154-8; doi: 10.1128/aem.44.5.1154-1158.1982.
52. Black RE, Levine MM, Clements ML, Hughes TP, Blaser MJ. Experimental *Campylobacter jejuni* infection in humans. J Infect Dis. 1988;157(3):472-9; doi: 10.1093/infdis/157.3.472.
53. Blaser MJ, Perez GP, Smith PF, Patton C, Tenover FC, Lastovica AJ, et al. Extraintestinal *Campylobacter jejuni* and *Campylobacter coli* infections: host factors and strain characteristics. J Infect Dis. 1986;153(3):552-9; doi: 10.1093/infdis/153.3.552.
54. Nachamkin I, Blaser MJ, Tompkins LS. *Campylobacter jejuni*: current status and future trends. Washington D.C.: American Society for Microbiology; 1992.
55. Tee W, Anderson B, Ross B, Dwyer B. Atypical campylobacters associated with gastroenteritis. J Clin Microbiol. 1987;25(7):1248-52; doi: 10.1128/jcm.25.7.1248-1252.1987.
56. Pettersen K, Moldal T, Gjerset B, Bergsjø B. The surveillance programme for *Campylobacter* spp. in broiler flocks in Norway 2020. https://www.vetinst.no/overvaking/campylobacter-fjorfe: Norwegian Veterinary Institute; 2021. Accessed June 20, 2022.
57. Jørgensen HJ, Hauge K, Lange H, Lyngstad TM, Heier BT. The Norwegian Zoonoses Report 2020. Norwegian Veterinary Institute; 2021.

58. Farrokh C, Jordan K, Auvray F, Glass K, Oppegaard H, Raynaud S, et al. Review of Shiga-toxin-producing *Escherichia coli* (STEC) and their significance in dairy production. Int J Food Microbiol. 2013;162(2):190-212; doi: 10.1016/j.ijfoodmicro.2012.08.008.
59. Bruyand M, Mariani-Kurkdjian P, Le Hello S, King L-A, Van Cauteren D, Lefevre S, et al. Paediatric haemolytic uraemic syndrome related to Shiga toxin-producing *Escherichia coli*, an overview of 10 years of surveillance in France, 2007 to 2016. Eurosurveillance. 2019;24(8):1800068; doi: 10.2807/1560-7917.ES.2019.24.8.1800068.
60. Nataro JP, Kaper JB. Diarrheagenic *Escherichia coli*. Clin Microbiol Rev. 1998;11(1):142-201; doi: 10.1128/CMR.11.1.142.
61. Riley LW, Remis RS, Helgerson SD, McGee HB, Wells JG, Davis BR, et al. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. N Engl J Med. 1983;308(12):681-5; doi: 10.1056/NEJM198303243081203.
62. Kim J-S, Lee M-S, Kim JH. Recent updates on outbreaks of Shiga toxin-producing *Escherichia coli* and its potential reservoirs. Frontiers in Cellular Infection Microbiology. 2020:273; doi: 10.3389/fcimb.2020.00273.
63. Fuller CA, Pellino CA, Flagler MJ, Strasser JE, Weiss AA. Shiga toxin subtypes display dramatic differences in potency. Infection immunity. 2011;79(3):1329-37; doi: 10.1128/IAI.01182-10.
64. Casjens SR, Hendrix RW. Bacteriophage lambda: Early pioneer and still relevant. Virology. 2015;479:310-30; doi: 10.1016/j.virol.2015.02.010.
65. Allison HE. Stx-phages: drivers and mediators of the evolution of STEC and STEC-like pathogens. Future Microbiology. 2007;2(2):165-74; doi: 10.2217/17460913.2.2.165.
66. Stayrook S, Jaru-Ampornpan P, Ni J, Hochschild A, Lewis M. Crystal structure of the λ repressor and a model for pairwise cooperative operator binding. Nature. 2008;452(7190):1022-5; doi: 10.1038/nature06831.
67. Krüger A, Lucchesi PM. Shiga toxins and stx phages: highly diverse entities. Microbiology. 2015;161(3):451-62; doi: 10.1099/mic.0.000003.
68. Joseph A, Cointe A, Mariani-Kurkdjian P, Rafat C, Hertig A. Shiga toxin-associated hemolytic uremic syndrome: a narrative review. Toxins (Basel). 2020;12(2):67; doi: 10.3390/toxins12020067.
69. Necel A, Bloch S, Nejman-Faleńczyk B, Dydecka A, Topka-Bielecka G, Węgrzyn A, et al. A Validation System for Selection of Bacteriophages against Shiga Toxin-Producing *Escherichia coli* Contamination. Toxins (Basel). 2021;13(9):644; doi: 10.3390/toxins13090644.

70. Fagerlund A, Aspholm M, Węgrzyn G, Lindbäck T. High diversity in the regulatory region of Stx-converting bacteriophage genomes 2. bioRxiv. 2021; doi: 10.1186/s12864-022-08428-5.
71. Karmali MA. Factors in the emergence of serious human infections associated with highly pathogenic strains of shiga toxin-producing *Escherichia coli*. Int J Med Microbiol. 2018;308(8):1067-72; doi: 10.1016/j.ijmm.2018.08.005.
72. Frank C, Werber D, Cramer JP, Askar M, Faber M, an der Heiden M, et al. Epidemic profile of Shiga-toxin–producing *Escherichia coli* O104: H4 outbreak in Germany. N Engl J Med. 2011;365(19):1771-80; doi: 10.1056/NEJMoa1106483.
73. Jaakkonen A, Castro H, Hallanvuo S, Ranta J, Rossi M, Isidro J, et al. Longitudinal Study of Shiga Toxin-Producing *Escherichia coli* and *Campylobacter jejuni* on Finnish Dairy Farms and in Raw Milk. Appl Environ Microbiol. 2019;85(7):e02910-18; doi: 10.1128/aem.02910-18.
74. Byrne L, Jenkins C, Launders N, Elson R, Adak G. The epidemiology, microbiology and clinical impact of Shiga toxin-producing *Escherichia coli* in England, 2009-2012. Epidemiology and Infection. 2015;143; doi: 10.1017/S0950268815000746.
75. Strachan NJ, Dunn GM, Locking ME, Reid TM, Ogden ID. *Escherichia coli* O157: burger bug or environmental pathogen? Int J Food Microbiol. 2006;112(2):129-37; doi: 10.1016/j.ijfoodmicro.2006.06.021.
76. Allerberger F, Friedrich AW, Grif K, Dierich MP, Dornbusch H-R, Mache CJ, et al. Hemolytic-uremic syndrome associated with enterohemorrhagic *Escherichia coli* O26: H infection and consumption of unpasteurized cow's milk. Int J Infect Dis. 2003;7(1):42-5; doi: 10.1016/S1201-9712(03)90041-5.
77. Liptakova A, Siegfried L, Rosocha J, Podracka L, Bogyiova E, Kotulova D. A family outbreak of haemolytic uraemic syndrome and haemorrhagic colitis caused by verocytotoxigenic *Escherichia coli* O157 from unpasteurised cow's milk in Slovakia. Clin Microbiol Infect. 2004;10(6):576-8; doi: 10.1111/j.1469-0691.2004.00900.x.
78. Jensen C, Ethelberg S, Gervelmeyer A, Nielsen E, Olsen KE, Mølbak K. First general outbreak of Verocytotoxin-producing *Escherichia coli* O157 in Denmark. Eurosurveillance. 2006;11(2):1-2; doi: 10.2807/esm.11.02.00597-en.
79. Centers for Disease Control and Prevention. *Escherichia coli* 0157: H7 infections in children associated with raw milk and raw colostrum from cows--California, 2006. Morb Mortal Wkly Rep. 2008;57(23):625-8.
80. De Schrijver K, Buvens G, Possé B, Van den Branden D, Oosterlynck O, De Zutter L, et al. Outbreak of verocytotoxin-producing *E. coli* O145 and O26 infections associated with the consumption of ice cream

produced at a farm, Belgium, 2007. Eurosurveillance. 2008;13(7):9-10; doi: 10.2807/ese.13.07.08041-en.
81. Rothschild M. Sally Jackson cheese confirmed as outbreak source. In. https://www.foodsafetynews.com/2010/12/sally-jackson-cheese-confirmed-as-outbreak-source/: Food Safety News; 2010. Accessed October 10, 2022.
82. Germinario C, Caprioli A, Giordano M, Chironna M, Gallone MS, Tafuri S, et al. Community-wide outbreak of haemolytic uraemic syndrome associated with Shiga toxin 2-producing *Escherichia coli* O26: H11 in southern Italy, summer 2013. Eurosurveillance. 2016;21(38):30343; doi: 10.2807/1560-7917.ES.2016.21.38.30343.
83. Treacy J, Jenkins C, Paranthaman K, Jorgensen F, Mueller-Doblies D, Anjum M, et al. Outbreak of Shiga toxin-producing *Escherichia col*i O157: H7 linked to raw drinking milk resolved by rapid application of advanced pathogen characterisation methods, England, August to October 2017. Eurosurveillance. 2019;24(16):1800191; doi: 10.2807/1560-7917.ES.2019.24.16.1800191.
84. Jones G, Lefèvre S, Donguy M-P, Nisavanh A, Terpant G, Fougère E, et al. Outbreak of Shiga toxin-producing *Escherichia coli* (STEC) O26 paediatric haemolytic uraemic syndrome (HUS) cases associated with the consumption of soft raw cow's milk cheeses, France, March to May 2019. Eurosurveillance. 2019;24(22):1900305; doi: 10.2807/1560-7917.ES.2019.24.22.1900305.
85. Kauppi K, Tatini S, Harrell F, Feng P. Influence of substrate and low temperature on growth and survival of verotoxigenic *Escherichia coli*. Food microbiology. 1996;13(5):397-405; doi: 10.1006/fmic.1996.0046.
86. Mamani Y, Quinto E, Simal-Gandara J, Mora M. Growth and survival of *Escherichia coli* O157: H7 in different types of milk stored at 4 C or 20 C. J Food Sci. 2003;68(8):2558-63; doi: 10.1111/j.1365-2621.2003.tb07061.x.
87. Heuvelink A, Bleumink B, Van Den Biggelaar F, Te Giffel M, Beumer R, De Boer E. Occurrence and survival of verocytotoxin-producing *Escherichia coli* O157 in raw cow's milk in The Netherlands. J Food Prot. 1998;61(12):1597-601; doi: 10.4315/0362-028X-61.12.1597.
88. Horn B, Pattis I, Soboleva T. Growth of microorganisms in raw milk: Evaluating the effect of chiller failure. New Zealand Food Safety Technical report No. 2020/04. New Zealand Food Safety; 2020.
89. Oliver SP, Jayarao BM, Almeida RA. Foodborne pathogens in milk and the dairy farm environment: food safety and public health implications. Foodborne Pathog Dis. 2005;2(2):115-29; doi: 10.1089/fpd.2005.2.115.
90. Artursson K, Schelin J, Thisted Lambertz S, Hansson I, Olsson Engvall E. Foodborne pathogens in unpasteurized milk in Sweden. Int J Food Microbiol. 2018;284:120-7; doi: 10.1016/j.ijfoodmicro.2018.05.015.

91. Steele M, McNab W, Poppe C, Griffiths M, Chen S, Degrandis S, et al. Survey of Ontario Bulk Tank Raw Milk for Food-Borne Pathogens. J Food Prot. 1997;60(11):1341-6; doi: 10.4315/0362-028x-60.11.1341.
92. De Reu K, Grijspeerdt K, Herman L. A Belgian survey of hygiene indicator bacteria and pathogenic bacteria in raw milk and direct marketing of raw milk farm products. J Food Saf. 2004;24(1):17-36; doi: 10.1111/j.1745-4565.2004.tb00373.x.
93. Jayarao BM, Donaldson SC, Straley BA, Sawant AA, Hegde NV, Brown J. A survey of foodborne pathogens in bulk tank milk and raw milk consumption among farm families in Pennsylvania. J Dairy Sci. 2006;89(7):2451-8; doi: 10.3168/jds.S0022-0302(06)72318-9.
94. Kalorey D, Warke S, Kurkure N, Rawool D, Barbuddhe S. *Listeria* species in bovine raw milk: A large survey of Central India. J Food Control. 2008;19(2):109-12; doi: 10.1016/j.foodcont.2007.02.006.
95. Vilar MJ, Yus E, Sanjuan ML, Diéguez F, Rodríguez-Otero J. Prevalence of and risk factors for *Listeria* species on dairy farms. J Dairy Sci. 2007;90(11):5083-8; doi: 10.3168/jds.2007-0213.
96. Waak E, Tham W, Danielsson-Tham M-L. Prevalence and Fingerprinting of *Listeria monocytogenes* Strains Isolated from Raw Whole Milk in Farm Bulk Tanks and in Dairy Plant Receiving Tanks. Appl Environ Microbiol. 2002;68(7):3366-70; doi: 10.1128/AEM.68.7.3366-3370.2002.
97. Silva J, Leite D, Fernandes M, Mena C, Gibbs PA, Teixeira P. *Campylobacter* spp. as a Foodborne Pathogen: A Review. Front Microbiol. 2011;2:200; doi: 10.3389/fmicb.2011.00200.
98. Johannessen G, Sekse C, Hopp P, Urdahl AM. Zoonotiske *E. coli* hos storfe, vol. 15. Norwegian Veterinary Institute; 2018.
99. Hill B, Smythe B, Lindsay D, Shepherd J. Microbiology of raw milk in New Zealand. Int J Food Microbiol. 2012;157(2):305-8; doi: 10.1016/j.ijfoodmicro.2012.03.031.
100. Marshall J, Soboleva T, Jamieson P, French N. Estimating bacterial pathogen levels in New Zealand bulk tank milk. J Food Prot. 2016;79(5):771-80; doi: 10.4315/0362-028X.JFP-15-230.
101. Heier BT, Hopp P, Mork J, Bergsjø B: The surveillance programme for *Salmonella* spp. in live animals, eggs and meat in Norway 2020. In. https://www.vetinst.no/overvaking/salmonella: Norwegian Veterinary Institute; 2021. Accessed October 30, 2022.
102. Moura A, Criscuolo A, Pouseele H, Maury MM, Leclercq A, Tarr C, et al. Whole genome-based population biology and epidemiological surveillance of *Listeria monocytogenes*. Nat Microbiol. 2016;2:16185-; doi: 10.1038/nmicrobiol.2016.185.
103. Wirth T, Falush D, Lan R, Colles F, Mensa P, Wieler LH, et al. Sex and virulence in *Escherichia coli*: an evolutionary perspective. Mol

Microbiol. 2006;60(5):1136-51; doi: 10.1111/j.1365-2958.2006.05172.x.
104. Salcedo C, Arreaza L, Alcala B, De La Fuente L, Vazquez J. Development of a multilocus sequence typing method for analysis of *Listeria monocytogenes* clones. J Clin Microbiol. 2003;41(2):757-62; doi: 10.1128/JCM.41.2.757-762.2003.
105. Ruppitsch W, Pietzka A, Prior K, Bletz S, Fernandez HL, Allerberger F, et al. Defining and evaluating a core genome multilocus sequence typing scheme for whole-genome sequence-based typing of *Listeria monocytogenes*. J Clin Microbiol. 2015;53(9):2869-76; doi: 10.1128/JCM.01193-15.
106. Cody AJ, Bray JE, Jolley KA, McCarthy ND, Maiden MCJ, Diekema DJ. Core Genome Multilocus Sequence Typing Scheme for Stable, Comparative Analyses of *Campylobacter jejuni* and *C. coli* Human Disease Isolates. Clinical Microbiology. 2017;55(7):2086-97; doi: doi:10.1128/JCM.00080-17.
107. Butcher H, Elson R, Chattaway MA, Featherstone C, Willis C, Jorgensen F, et al. Whole genome sequencing improved case ascertainment in an outbreak of Shiga toxin-producing *Escherichia coli* O157 associated with raw drinking milk. Epidemiol Infect. 2016;144(13):2812-23; doi: 10.1017/S0950268816000509.
108. Blanc DS, Magalhães B, Koenig I, Senn L, Grandbastien B. Comparison of Whole Genome (wg-) and Core Genome (cg-) MLST (BioNumerics(TM)) Versus SNP Variant Calling for Epidemiological Investigation of *Pseudomonas aeruginosa*. Front Microbiol. 2020;11:1729; doi: 10.3389/fmicb.2020.01729.
109. Møretrø T, Schirmer BC, Heir E, Fagerlund A, Hjemli P, Langsrud S. Tolerance to quaternary ammonium compound disinfectants may enhance growth of *Listeria monocytogenes* in the food industry. Int J Food Microbiol. 2017;241:215-24; doi: 10.1016/j.ijfoodmicro.2016.10.025.
110. Massip C, Guet-Revillet H, Grare M, Sommet A, Dubois D. Enhanced culture recovery of *Campylobacter* with modified Cary-Blair medium: A practical field experience. J Microbiol Methods. 2018;149:53-4; doi: 10.1016/j.mimet.2018.05.001.
111. Eichhorn I, Heidemanns K, Semmler T, Kinnemann B, Mellmann A, Harmsen D, et al. Highly virulent non-O157 enterohemorrhagic *Escherichia coli* (EHEC) serotypes reflect similar phylogenetic lineages, providing new insights into the evolution of EHEC. Appl Environ Microbiol. 2015;81(20):7041-7; doi: 10.1128/AEM.01921-15.
112. Sánchez S, Llorente MT, Echeita MA, Herrera-León S. Development of three multiplex PCR assays targeting the 21 most clinically relevant serogroups associated with Shiga toxin-producing *E. coli* infection in

humans. PLoS One. 2015;10(1):e0117660-e; doi: 10.1371/journal.pone.0117660.
113. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, et al. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol. 2012;19(5):455-77; doi: 10.1089/cmb.2012.0021.
114. Dijkshoorn L, Ursing B, Ursing J. Strain, clone and species: comments on three basic concepts of bacteriology. J Med Microbiol. 2000;49(5):397-401; doi: 10.1099/0022-1317-49-5-397.
115. Zamudio R, Haigh RD, Ralph JD, De Ste Croix M, Tasara T, Zurfluh K, et al. Lineage-specific evolution and gene flow in *Listeria monocytogenes* are independent of bacteriophages. Environ Microbiol. 2020;22(12):5058-72; doi: 10.1111/1462-2920.15111.
116. Wang Y, Pettengill JB, Pightling A, Timme R, Allard M, Strain E, et al. Genetic diversity of *Salmonella* and *Listeria* isolates from food facilities. J Food Prot. 2018;81(12):2082-9; doi: 10.4315/0362-028X.JFP-18-093.
117. Allard MW, Strain E, Rand H, Melka D, Correll WA, Hintz L, et al. Whole genome sequencing uses for foodborne contamination and compliance: discovery of an emerging contamination event in an ice cream facility using whole genome sequencing. Infect Genet Evol. 2019;73:214-20; doi: 10.1016/j.meegid.2019.04.026.
118. Pightling AW, Pettengill JB, Luo Y, Baugher JD, Rand H, Strain E. Interpreting whole-genome sequence analyses of foodborne bacteria for regulatory applications and outbreak investigations. Front Microbiol. 2018;9:1482; doi: 10.3389/fmicb.2018.0148.
119. Gerner-Smidt P, Besser J, Concepción-Acevedo J, Folster JP, Huffman J, Joseph LA, et al. Whole genome sequencing: bridging one-health surveillance of foodborne diseases. Frontiers in Public Health. 2019;7:172; doi: 10.3389/fpubh.2019.00172.
120. Schürch A, Arredondo-Alonso S, Willems R, Goering R. Whole genome sequencing options for bacterial strain typing and epidemiologic analysis based on single nucleotide polymorphism versus gene-by-gene–based approaches. Clin Microbiol Infect. 2018;24(4):350-4; doi: 10.1016/j.cmi.2017.12.016.
121. Leclair RM, McLean SK, Dunn LA, Meyer D, Palombo EA. Investigating the effects of time and temperature on the growth of *Escherichia coli* O157: H7 and *Listeria monocytogenes* in raw cow's milk based on simulated consumer food handling practices. Int J Environ Res Public Health. 2019;16(15):2691; doi: 10.3390/ijerph16152691.
122. Rangel JM, Sparling PH, Crowe C, Griffin PM, Swerdlow DL. Epidemiology of *Escherichia coli* O157: H7 outbreaks, united states, 1982–2002. Emerging Infect Dis. 2005;11(4):603; doi: 10.3201/eid1104.040739.

123. Massa S, Goffredo E, Altieri C, Natola K. Fate of *Escherichia coli* O157: H7 in unpasteurized milk stored at 8 C. Lett Appl Microbiol. 1999;28(1):89-92; doi: 10.1046/j.1365-2672.1999.00408.x.
124. Fremaux B, Prigent-Combaret C, Vernozy-Rozand C. Long-term survival of Shiga toxin-producing *Escherichia coli* in cattle effluents and environment: an updated review. Vet Microbiol. 2008;132(1-2):1-18; doi: 10.1016/j.vetmic.2008.05.015.
125. Necel A, Bloch S, Nejman-Faleńczyk B, Grabski M, Topka G, Dydecka A, et al. Characterization of a bacteriophage, vB_Eco4M-7, that effectively infects many *Escherichia coli* O157 strains. Sci Rep. 2020;10(1):3743; doi: 10.1038/s41598-020-60568-4.
126. Beutin L, Steinrück H, Krause G, Steege K, Haby S, Hultsch G, et al. Comparative evaluation of the Ridascreen® Verotoxin enzyme immunoassay for detection of Shiga-toxin producing strains of *Escherichia coli* (STEC) from food and other sources. J Appl Microbiol. 2007;102(3):630-9; doi: 10.1111/j.1365-2672.2006.03139.x.
127. Shimizu K, Asahara T, Nomoto K, Tanaka R, Hamabata T, Ozawa A, et al. Development of a lethal Shiga toxin-producing *Escherichia coli*-infection mousemodel using multiple mitomycin C treatment. Microb Pathog. 2003;35(1):1-9; doi: 10.1016/S0882-4010(03)00065-2.
128. Muniesa M, Blanco JE, De Simón M, Serra-Moreno R, Blanch AR, Jofre J. Diversity of *stx2* converting bacteriophages induced from Shiga-toxin-producing *Escherichia coli* strains isolated from cattle. Microbiology. 2004;150(9):2959-71; doi: 10.1099/mic.0.27188-0.
129. Pierzynowska K, Jasińska W, Cyske Z, Bunikowska M, Droczek R, Węgrzyn G. Effects of some commonly used drinks on induction of Shiga toxin-converting prophage in *Escherichia coli*. J Consum Prot Food Saf. 2018;13(2):125-9; doi: 10.1007/s00003-018-1155-z.
130. Claeys WL, Cardoen S, Daube G, De Block J, Dewettinck K, Dierick K, et al. Raw or heated cow milk consumption: Review of risks and benefits. J Food control. 2013;31(1):251-62; doi: 10.1016/j.foodcont.2012.09.035.
131. Maldonado YA, Glode MP, Bhatia J, Brady MT, Byington CL, Davies HD, et al. Consumption of raw or unpasteurized milk and milk products by pregnant women and children. Pediatrics. 2014;133(1):175-9; doi: 10.1542/peds.2013-3502.
132. Panel on Biological Hazards, European Food Safety Authority. Scientific opinion on the public health risks related to the consumption of raw drinking milk. EFSA J. 2015;13(1):3940; doi: 10.2903/j.efsa.2015.3940.
133. Lovett J, Francis DW, Hunt J. Isolation of *Campylobacter jejuni* from raw milk. Appl Environ Microbiol. 1983;46(2):459-62; doi: 10.1128/aem.46.2.459-462.1983.

134. McManus C, Lanier JM. *Salmonella*, *Campylobacter jejuni*, and *Yersinia enterocolitica* in Raw Milk. J Food Prot. 1987;50(1):51-5; doi: 10.4315/0362-028x-50.1.51
135. Davidson R, Sprung D, Park C, Rayman M. Occurrence of *Listeria monocytogenes*, *Campylobacter* spp. and *Yersinia enterocolitica* in Manitoba raw milk. Canadian Institute of Food Science and Technology Journal. 1989;22(1):70-4; doi: 10.1016/S0315-5463(89)70304-7.
136. Jayarao BM, Henning DR. Prevalence of Foodborne Pathogens in Bulk Tank Milk. J Dairy Sci. 2001;84(10):2157-62; doi: 10.3168/jds.S0022-0302(01)74661-9.
137. Slade P, Collins-Thompson D, Fletcher F. Incidence of *Listeria* species in Ontario raw milk. Canadian Institute of Food Science and Technology Journal. 1988;21(4):425-9; doi: 10.1016/S0315-5463(88)70980-3.
138. Van Kessel JS, Karns JS, Gorski L, McCluskey BJ, Perdue ML. Prevalence of *Salmonellae*, *Listeria monocytogenes*, and Fecal Coliforms in Bulk Tank Milk on US Dairies. J Dairy Sci. 2004;87(9):2822-30; doi: 10.3168/jds.S0022-0302(04)73410-4.
139. Murinda S, Nguyen L, Ivey S, Gillespie B, Almeida R, Draughon F, et al. Prevalence and molecular characterization of *Escherichia coli* O157: H7 in bulk tank milk and fecal samples from cull cows: a 12-month survey of dairy farms in east Tennessee. J Food Prot. 2002;65(5):752-9; doi: 10.4315/0362-028X-65.5.752.
140. de Koning K, Slaghuis B, van der Vorst Y. Robotic milking and milk quality. Effects on bacterial counts - somatic cell counts - freezing point and free fatty acids. Ital J Anim Sci. 2003;2(4):291-9; doi: 10.4081/ijas.2003.291.
141. Klungel G, Slaghuis B, Hogeveen H. The effect of the introduction of automatic milking systems on milk quality. J Dairy Sci. 2000;83(9):1998-2003; doi: 10. 3168/jds.S0022-0302(00)75077-6.
142. van der Vorst Y, Hogeveen H: Automatic milking systems and milk quality in the Netherlands. In: Robotic milking: Proceedings of the International Symposium held in Lelystad, The Netherlands, 17-19 August, 2000. Wageningen Pers; 2000: 73-82.
143. Rasmussen MD, Bjerring M, Justesen P, Jepsen L. Milk quality on Danish farms with automatic milking systems. J Dairy Sci. 2002;85(11):2869-78; doi: 10.3168/jds.S0022-0302(02)74374-9.
144. Rasmussen MD, Blom JY, Nielsen LAH, Justesen P. Udder health of cows milked automatically. Livest Prod Sci. 2001;72(1-2):147-56; doi: 10.1016/S0301-6226(01)00275-5.
145. Van der Vorst Y, Ouweltjes W: Milk quality and automatic milking; a risk inventory, vol. 28. Lelystad, The Netherlands: Research Institute for Animal Husbandry; 2003.

146. Kruip T, Morice H, Robert M, Ouweltjes W. Robotic milking and its effect on fertility and cell counts. J Dairy Sci. 2002;85(10):2576-81; doi: 10.3168/jds.S0022-0302(02)74341-5.
147. Hovinen M, Rasmussen MD, Pyörälä S. Udder health of cows changing from tie stalls or free stalls with conventional milking to free stalls with either conventional or automatic milking. J Dairy Sci. 2009;92(8):3696-703; doi: 10.3168/jds.2008-1962.
148. Johansson M, Lundh Å, de Vries R, Sjaunja KS. Composition and enzymatic activity in bulk milk from dairy farms with conventional or robotic milking systems. J Dairy Res. 2017;84(2):154-8; doi: 10.1017/S0022029917000140.
149. Tousova R, Duchacek J, Stadnik L, Ptacek M, Beran J. The comparison of milk production and quality in cows from conventional and automatic milking systems. J Cent Eur Agri. 2014;15(4):100-14; doi: 10.5513/JCEA01/15.4.1515.
150. Petrovska S, Jonkus D: Milking technology influence on dairy cow milk productivity and quality. 13th International Scientific Conference, Engineering for Rural Development Proceedings, vol. 13. Jelgava, Latvia; 2014: p.29-30.
151. Hogenboom JA, Pellegrino L, Sandrucci A, Rosi V, D'Incecco P. Invited review: Hygienic quality, composition, and technological performance of raw milk obtained by robotic milking of cows. J Dairy Sci. 2019;102(9):7640-54; doi: 10.3168/jds.2018-16013.
152. Derakhshani H, Fehr KB, Sepehri S, Francoz D, De Buck J, Barkema HW, et al. Invited review: Microbiota of the bovine udder: Contributing factors and potential implications for udder health and mastitis susceptibility. J Dairy Sci. 2018;101(12):10605-25; doi: 10.3168/jds.2018-14860.
153. Svennersten-Sjaunja KM, Pettersson G. Pros and cons of automatic milking in Europe. J Anim Sci. 2008;86(13):37-46; doi: 10.2527/jas.2007-0527.
154. Galama PJ, Ouweltjes W, Endres MI, Sprecher JR, Leso L, Kuipers A, et al. Symposium review: Future of housing for dairy cattle. J Dairy Sci. 2020;103(6):5759-72; doi: 10.3168/jds.2019-17214.
155. Aytekin İ, Altay Y, Boztepe S, Keskin İ, Zulkadir U. The effect of body cleanliness (hygiene) score on some criteria used in the detection milk quality in dairy cattle. Large Anim Rev. 2021;27(2):69-74.
156. Hauge S, Kielland C, Ringdal G, Skjerve E, Nafstad O. Factors associated with cattle cleanliness on Norwegian dairy farms. J Dairy Sci. 2012;95(5):2485-96; doi: 10.3168/jds.2011-4786.
157. Neja W, Bogucki M, Jankowska M, Sawa A. Effect of cow cleanliness in different housing systems on somatic cell count in milk. Acta Vet Brno. 2016;85(1):55-61; doi: 10.2754/avb201685010055.

158. Schreiner D, Ruegg P. Relationship between udder and leg hygiene scores and subclinical mastitis. J Dairy Sci. 2003;86(11):3460-5; doi: 10.3168/jds.S0022-0302(03)73950-2.
159. Liao J, Guo X, Weller DL, Pollak S, Buckley DH, Wiedmann M, et al. Nationwide genomic atlas of soil-dwelling *Listeria* reveals effects of selection and population ecology on pangenome evolution. Nat Microbiol. 2021;6(8):1021-30; doi: 10.1038/s41564-021-00935-7.
160. Ragon M, Wirth T, Hollandt F, Lavenir R, Lecuit M, Le Monnier A, et al. A new perspective on *Listeria monocytogenes* evolution. PLoS Pathog. 2008;4(9):e1000146; doi: 10.1371/journal.ppat.1000146.
161. Maury MM, Tsai YH, Charlier C, Touchon M, Chenal-Francisque V, Leclercq A, et al. Uncovering *Listeria monocytogenes* hypervirulence by harnessing its biodiversity. Nat Genet. 2016;48(3):308-13; doi: 10.1038/ng.3501.
162. Chenal-Francisque V, Lopez J, Cantinelli T, Caro V, Tran C, Leclercq A, et al. Worldwide distribution of major clones of *Listeria monocytogenes*. Emerg Infect Dis. 2011;17(6):1110-2; doi: 10.3201/eid1706.101778.
163. Bergholz TM, Shah MK, Burall LS, Rakic-Martinez M, Datta AR. Genomic and phenotypic diversity of *Listeria monocytogenes* clonal complexes associated with human listeriosis. Appl Microbiol Biotechnol. 2018;102(8):3475-85; doi: 10.1007/s00253-018-8852-5.
164. Dreyer M, Aguilar-Bultet L, Rupp S, Guldimann C, Stephan R, Schock A, et al. *Listeria monocytogenes* sequence type 1 is predominant in ruminant rhombencephalitis. Sci Rep. 2016;6:36419; doi: 10.1038/srep36419.
165. Nightingale KK, Schukken YH, Nightingale CR, Fortes ED, Ho AJ, Her Z, et al. Ecology and transmission of *Listeria monocytogenes* infecting ruminants and in the farm environment. Appl Environ Microbiol. 2004;70(8):4458-67; doi: 10.1128/AEM.70.8.4458-4467.2004.
166. Latorre AA, Van Kessel JA, Karns JS, Zurakowski MJ, Pradhan AK, Boor KJ, et al. Increased *in vitro* adherence and on-farm persistence of predominant and persistent *Listeria monocytogenes* strains in the milking system. Appl Environ Microbiol. 2011;77(11):3676-84; doi: 10.1128/AEM.02441-10.
167. Fonnesbech Vogel B, Huss HH, Ojeniyi B, Ahrens P, Gram L. Elucidation of *Listeria monocytogenes* contamination routes in cold-smoked salmon processing plants detected by DNA-based typing methods. Appl Environ Microbiol. 2001;67(6):2586-95; doi: 10.1128/AEM.67.6.2586-2595.2001.
168. Fagerlund A, Langsrud S, Møretrø T. Microbial diversity and ecology of biofilms in food industry environments associated with *Listeria monocytogenes* persistence. Curr Opin Food Sci. 2021;37:171-8; doi: 10.1016/j.cofs.2020.10.015.

169. Latorre AA, Van Kessel JS, Karns JS, Zurakowski MJ, Pradhan AK, Boor KJ, et al. Biofilm in milking equipment on a dairy farm as a potential source of bulk tank milk contamination with *Listeria monocytogenes*. J Dairy Sci. 2010;93(6):2792-802; doi: 10.3168/jds.2009-2717.
170. Borucki MK, Peppin JD, White D, Loge F, Call DR. Variation in Biofilm Formation among Strains of *Listeria monocytogenes*. Appl Environ Microbiol. 2003;69(12):7336-42; doi: 10.1128/AEM.69.12.7336-7342.2003.
171. Fagerlund A, Langsrud S, Schirmer BCT, Møretrø T, Heir E. Genome analysis of *Listeria monocytogenes* sequence type 8 strains persisting in salmon and poultry processing environments and comparison with related strains. PLoS One. 2016;11(3):e0151117; doi: 10.1371/journal.pone.0151117.
172. Knabel SJ, Reimer A, Verghese B, Lok M, Ziegler J, Farber J, et al. Sequence typing confirms that a predominant *Listeria monocytogenes* clone caused human listeriosis cases and outbreaks in Canada from 1988 to 2010. J Clin Microbiol. 2012;50(5):1748-51; doi: 10.1128/JCM.06185-11.
173. Mammina C, Parisi A, Guaita A, Aleo A, Bonura C, Nastasi A, et al. Enhanced surveillance of invasive listeriosis in the Lombardy region, Italy, in the years 2006-2010 reveals major clones and an increase in serotype 1/2a. BMC Infect Dis. 2013;13(1):1-8; doi: 10.1186/1471-2334-13-152.
174. Fagerlund A, Langsrud S, Møretrø T. In-depth longitudinal study of *Listeria monocytogenes* ST9 isolates from the meat processing industry: Resolving diversity and transmission patterns using whole-genome sequencing. Appl Environ Microbiol. 2020;86(14):e00579-20; doi: 10.1128/AEM.00579-20.
175. Martín B, Perich A, Gómez D, Yangüela J, Rodríguez A, Garriga M, et al. Diversity and distribution of *Listeria monocytogenes* in meat processing plants. Food Microbiol. 2014;44:119-27; doi: 10.1016/j.fm.2014.05.014.
176. Félix B, Feurer C, Maillet A, Guillier L, Boscher E, Kerouanton A, et al. Population genetic structure of *Listeria monocytogenes* strains isolated from the pig and pork production chain in France. Front Microbiol. 2018;9:684; doi: 10.3389/fmicb.2018.00684.
177. Maury MM, Bracq-Dieye H, Huang L, Vales G, Lavina M, Thouvenot P, et al. Hypervirulent *Listeria monocytogenes* clones' adaptation to mammalian gut accounts for their association with dairy products. Nat Commun. 2019;10(1):2488; doi: 10.1038/s41467-019-10380-0.
178. Kim SW, Haendiges J, Keller EN, Myers R, Kim A, Lombard JE, et al. Genetic diversity and virulence profiles of *Listeria monocytogenes* recovered from bulk tank milk, milk filters, and milking equipment from dairies in the United States (2002 to 2014). PLoS One. 2018;13(5):e0197053; doi: 10.1371/journal.pone.0197053.

179. Terentjeva M, Šteingolde Z, Meistere I, Elferts D, Avsejenko J, Streikiša M, et al. Prevalence, genetic diversity and factors associated with distribution of *Listeria monocytogenes* and other *Listeria* spp. in cattle farms in Latvia. Pathogens. 2021;10:851; doi: 10.3390/pathogens10070851.
180. Uelze L, Grützke J, Borowiak M, Hammerl JA, Juraschek K, Deneke C, et al. Typing methods based on whole genome sequencing data. One Health Outlook. 2020;2(1):1-19; doi: 10.1186/s42522-020-0010-1.
181. Mufandaedza J, Viljoen B, Feresu S, Gadaga T. Antimicrobial properties of lactic acid bacteria and yeast-LAB cultures isolated from traditional fermented milk against pathogenic *Escherichia coli* and *Salmonella enteritidis* strains. Int J Food Microbiol. 2006;108(1):147-52; doi: 10.1016/j.ijfoodmicro.2005.11.005.
182. Robinson D. Infective dose of *Campylobacter jejuni* in milk. Br Med J (Clin Res Ed). 1981;282(6276):1584; doi: 10.1136/bmj.282.6276.1584.
183. Marklinder I, Lindblad M, Eriksson L, Finnson A, Lindqvist R. Home storage temperatures and consumer handling of refrigerated foods in Sweden. J Food Prot. 2004;67(11):2570-7; doi: 10.4315/0362-028X-67.11.2570.

13 Scientific papers I-III

Paper I

Received: 10 January 2022 | Revised: 25 February 2022 | Accepted: 28 February 2022

DOI: 10.1111/jam.15512

Journal of Applied Microbiology sfam

ORIGINAL ARTICLE

The prevalence 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

Correspondence
Toril Lindbäck, Department of Paraclinical Sciences, Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Ås, Norway.
Email: toril.lindback@nmbu.no

Abstract

Aims: This study explored how dairy farm operating systems with free-stall or tie-stall housing and cow hygiene score influence the occurrence of zoonotic bacteria in raw milk.

Methods and Results: Samples from bulk tank milk (BTM), milk filters, faeces, feed, teats and teat milk were collected from 11 farms with loose housing and seven farms with tie-stall housing every second month over a period of 11 months and analysed for the presence of STEC by culturing combined with polymerase chain reaction and for *Campylobacter* spp. and *L. monocytogenes* by culturing only. *Campylobacter* spp., *L. monocytogenes* and STEC were present 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 BTM samples. Four STEC isolates carried the *eae* gene, which is linked to the capacity to cause severe human disease. *L. monocytogenes* were detected more frequently in loose housing herds compared with tie-stalled herds in faeces ($p = 0.02$) and feed ($p = 0.03$), and *Campylobacter* spp. were detected more frequently in loose housing herds in faeces ($p < 0.01$) and teat swabs ($p = 0.03$). An association between cow hygiene score and detection of *Campylobacter* spp. in teat milk was observed ($p = 0.03$).

Conclusion: Since some samples collected from loose housing systems revealed a significantly higher ($p < 0.05$) content of *L. monocytogenes* and *Campylobacter* spp. than samples collected from tie-stalled herds, the current study suggests that the type of housing system may 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 important knowledge for evaluating the risk of drinking unpasteurized milk.

KEYWORDS

Agriculture, *Campylobacter*, Food safety, *Listeria*, STEC (Shiga toxin-producing *E. coli*)

INTRODUCTION

Pasteurization of cow milk has been a practice in Europe since the 1880s 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) (Regulation [EC] No 853/2004). 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 with 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 sectors 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 faeces. 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 (Chlebicz & Śliżewska, 2018; Roberts & Wiedmann, 2003; Sapountzis et al., 2020). Other studies and reports highlight these bacteria as hazards related to consumption of UPM (Artursson et al., 2018; Castro et al., 2017; De Buyser et al., 2001; Jaakkonen et al., 2019; Langer et al., 2012; Lundén et al., 2004). *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 from dairy farms show genetic similarity to isolates from human campylobacteriosis cases (An et al., 2018). *Listeria 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 diarrhoea to bloody diarrhoea and haemolytic uremic syndrome (HUS). The World Health Organization (WHO) has estimated that 10% of patients with STEC infections develop HUS with a mortality rate of 2%–5%. The Shiga toxin (Stx) is the major virulence factor of STEC 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 STEC involved in enteropathogenic and enterohaemorrhagic diarrhoea (Donnenberg et al., 1993; Schmidt, 2010).

Listeria 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 *L. monocytogenes* contamination of raw milk from cows with *Listeria* mastitis may also occur but contamination via the milking instruments, where this pathogen can persist on surfaces, is probably a more relevant route of transmission to raw milk (Borucki et al., 2005; Yoshida et al., 1998). Other studies have shown that *L. monocytogenes* is able to propagate in refrigerated milk during storage (Artursson et al., 2018; Castro et al., 2017, 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 their ability to cause disease in humans (Epps et al., 2013). *Salmonella* spp. were not included in this study as the Norwegian Veterinary Institute performs continuous *Salmonella* surveillance and estimate a prevalence below 0.1% in the Norwegian cattle population. Most (78%–80%) of the human salmonellosis cases in Norway are acquired abroad and are rarely caused by domestically produced food (Norwegian Veterinary Institute, 2019).

Automatic milking systems (AMS) with robotic milking were introduced to European dairy farms in the early 1990s, (Cogato et al., 2021; Jacobs & Siegford, 2012). Since 2000, AMS have become common installations in Norwegian dairy farms and, today, more than 50% of the milk produced in Norway, originates from farms using milking robots (Hansen et al., 2019; Nørstebø et al., 2018). AMS is common in farms with large herds and loose housing where significant contact occurs between animals. This can lead to more problems with faecal contamination and cow cleanliness than experienced in tie-stall housing systems (Hovinen et al., 2009; Hovinen & Pyörälä, 2011). Other studies have investigated possible connections between farm operational systems and total bacterial count in bulk tank milk (BTM) (de Koning et al., 2003; Klungel et al., 2000; Rasmussen et al., 2001, 2002; van der Vorst & Hogeveen, 2000; Van der Vorst & Ouweltjes, 2003), but to our knowledge; there is limited knowledge on how the transition from tie-stall to loose housing influence the occurrence of zoonotic bacteria in the farm environment and in BTM. 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 systems. The relationship between herd hygienic status and the presence of *L. monocytogenes*, *Campylobacter* spp., and STEC in the farm samples was also evaluated.

MATERIALS AND METHODS

To assess the risk associated with consuming unpasteurized milk in Norway, aseptic samples of 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 faeces, 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, located within 100 km from Oslo, in southeast of Norway were randomly selected from a registry (Brønnøysundregisteret) where all Norwegian dairy-herds are registered. The milk produced at the farms is used for commercial production of drinking milk, cream, cheese, sour cream, yoghurt and other dairy products. Seven of the herds had tie-stall housing where the cows are tied up in individual bedding, feeding, and milking stalls. The tie-stall farms use conventional milking systems with manual application performed by an operator, usually the farmer, at specific times of the day. Eleven of the herds had loose housing where cows share a pen with common bedding, feeding and grooming area. In nine of the loose housed herds, the cows had access to an AMS which they enter voluntarily at any time of the day. One farm had loose housing with an integrated milking parlour operated by the farmer, and there was also a loose housing farm with milking performed on a carousel operated by the farmer. All herds have individual teat washing before milking, and some farms uses post milking teat dipping/spraying to secure udder hygiene. The milk is cooled (4°C) and stored in an on-farm bulk tank before transported to the dairy within 2–3 days. 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). The loose housed cows had access to an outdoor pasture for a minimum of 8 weeks during the sampling period, and the tie-stalled cows a minimum of 16 weeks. To account for seasonal variations in pathogen occurrence, each farm was sampled six times over a period of 11 months, with some exceptions due to Covid19 restrictions and other technical issues, resulting in variation in total number of samples from the farms. 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 BTM, milk filter, faeces, 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 they were immediately placed in a cooling bag (32 l, 50 × 33 × 41 cm) containing three to four freeze elements. The microbiological analyses were initiated within 6 h after sample collection.

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 h 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.

Faeces

Fresh faecal samples were collected from the floor at 5–10 different places in each animal house and pooled into a sterile stomacher bag to a total amount of minimum 100 g. 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 5 to 10 different

locations of the feed alley and pooled into a sterile stomacher bag. 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 <50 animals or more than 100, the numbers of sampled animals were limited to 5 and 10 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 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. Three distinct zones of the cow; the udder, lower portions of the hind limbs and upper portions of the hind limbs/flanks, were assessed according to a point scale 0–3, 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 and 3 was filthy, confluent plaques of manure (Cook, 2002). Further, the score from the three zones were added together to a total score between zero and nine for each cow, and a mean score was calculated for the herd at each visit. A lower score indicates better hygiene.

Isolation of *L. monocytogenes*

The samples (25 g BTM, 1/3 milk filter, 10 g faeces, 10 g feed, 5 ml teat swab solution and 5 ml teat milk) were cultured for *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) at 30°C for 24 h, followed by enrichment in full selectivity Fraser broth (Oxoid) at 37°C for 48 h. Cultures from the Fraser enrichments were plated on 'Agar Listeria according to Agosti and Ottaviani' (ALOA) and incubated at 37°C for 24–48 h. The concentration of *L. monocytogenes* in BTM and teat milk was assessed by plating 100 µl of the samples directly on ALOA. The plates were incubated at 37°C for 24–48 h before enumeration. Presumptive *L. monocytogenes* colonies from ALOA plates were confirmed after identification of beta-haemolytic, 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), faeces (10 g), teat swab solutions (5 ml) and teat milk samples (5 ml) were transferred into Bolton broth (Oxoid) for enrichment in a 1:10 ratio and then incubated at 37°C for 48 h in a 5% CO_2 atmosphere. The samples were further plated on selective agar mCCDA (modified charcoal cefoperazone deoxycholate agar; Oxoid) and incubated at 42°C for 48 h in a 5% CO_2 atmosphere. For enumeration, 100 µl of BTM and teat milk were plated on mCCDA and incubated for 48 h at 37°C. Presumptive *Campylobacter* colonies were confirmed as *Campylobacter* spp. when they were catalase and oxidase positive and appeared as motile s-shaped rods under phase-contrast microscopy.

Identification of STEC in samples

For enrichment of *E. coli* from either 25 ml BTM (100 samples), 1/3 of a milk filter (100 samples), or 10 g of faeces (98 samples), the samples were added to 225, 200 or 90 ml, respectively, of modified Tryptone Soya Broth (mTSB) (Oxoid), supplemented with novobiocin (16 µg/ml) according to ISO/TS 13136:2012, and incubated at 37°C for 24 h. Each pre-culture was then divided into two parts: one part containing 1 ml that was pelleted at 12,000 *g* 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 DNeasy® Blood and Tissue kit (Qiagen), 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 either *stx1*, *stx2* or *eae* were spread on CHROMagar STEC plates (CHROMagar Microbiology) by using an inoculation loop of 1 µl and incubated at 37°C for 24 h. CHROMagar STEC differentiate between STEC (mauve/pink colonies) and other Enterobacteriacae (blue colonies) and inhibits growth of Gram-positive bacteria. Three mauve/pink colonies

TABLE 1 Prevalence of *Listeria monocytogenes* in dairy farm samples

Sampling	BTM	MF	Faeces	Silage	Teat swab	Teat milk	Total
Aug./Sept.	0/18 (0)	0/17 (0)	5/16 (31)	4/18 (22)	0/17 (0)		9/86 (10)
Nov./Dec.	0/18 (0)	4/16 (25)	6/18 (33)	6/18 (33)	1/18 (6)		17/88 (19)
Jan	0/18 (0)	2/17 (12)	4/18 (22)	10/18 (56)	1/18 (6)	0/18 (0)	17/107 (16)
Feb./Mar.	0/18 (0)	1/13 (8)	7/18 (39)	6/18 (33)	1/18 (6)	0/18 (0)	15/103 (15)
May	0/14 (0)	2/14 (14)	2/14 (14)	3/14 (21)	2/14 (14)	0/14 (0)	9/84 (11)
June	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)

Note: Prevalence of bulk tank milk- (BTM), milk filter- (MF), faeces-, silage-, teat swab- and teat milk-samples positive for *Listeria monocytogenes*. The numbers given are positive samples/total samples (%). The samples were collected at six different time points between August 2019 and July 2020.

from each CHROMagar STEC plate were transferred to Sorbitol MacConkey Agar (SMAC) (Oxoid) plates for two purposes; to achieve single colonies for further testing by PCR and for direct identification of STEC of serotype O157 which grow with beige colonies on SMAC. Resulting single colonies isolated from the three SMAC plates 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 (Table S1). The amplification reactions were run separately for *stx1* and *stx2*, and 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 and autoclaved water as negative control. The PCR fragments were visualized by agarose gel electrophoresis.

Single colonies from the pure cultures on SMAC (transferred from CHROMagar STEC) were dissolved in 100 μl of autoclaved H_2O, heated for 99°C for 10 min, and 1 μl of this sample was examined for the presence of *stx1*, *stx2* and *eae* using the primers listed in Table S1. The EaeR primer were designed to detect the Alpha, Beta, Gamma, Zeta, Theta and the Delta versions of *eae*. Attempts to determine the serotype of the STECs by PCR were performed using the primers and conditions described by Sánchez et al. (2015). The *E. coli* isolates used as positive controls were of serotype O103 and O157, and autoclaved water was used as negative control.

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) 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 and the repeated sampling was generally taken into account by including the herd as a random variable in the regression models. Seasonal variation in the occurrence of pathogens in samples were taken account of by including visits as a fixed variable in the regression models. The effect of hygiene scores on the occurrence of pathogenic bacteria in the different samples was analysed by including visit as a random variable to account for repeated observations. Odds ratios (OR) are given to describe the effect of the binary variables (e.g. tie-stall versus loose housing) and β-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 (faeces and fodder). Statistical significance was defined as $p < 0.05$.

Results

Prevalence of *L. monocytogenes*, *Campylobacter* spp. and STEC on Norwegian dairy farms

Listeria monocytogenes was isolated from 79 of 556 samples (14%) and the distribution of positive samples is shown in Table 1. None of the 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.

Silage or silage mixture samples collected in January revealed a higher occurrence of *L. monocytogenes* than those collected in August–September ($\beta = 1.48$, $p = 0.03$) and June ($\beta = 1.60$, $p = 0.03$). The other sample types showed no seasonal differences (Table S2).

The prevalence of *Campylobacter* spp. was 20% among a total of 455 tested samples (Table 2). *Campylobacter* spp. were not detected by direct plating of BTM and teat milk on mCCDA agar. However, *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 after enrichment in Bolton broth. Among faecal samples, 68% were positive for *Campylobacter* spp. All farms had at least one *Campylobacter* spp. positive faecal 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 faeces during visit two/November–December and during visit five/May (Table 2, Table S3).

The frequency of BTM-samples, milk filter-samples and faecal-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 faeces 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 of 65 isolates were positive for either *stx1 or stx2,* or a combination of *stx1* and *stx2* and were, therefore, regarded as STECs (Table 4). None of the 19 *stx* positive isolates presented beige colonies on SMAC plates, indicating other serotypes than O157:H7. Out of 298 tested samples, STEC were isolated from 6% (19) of the samples. Muliplex PCR, targeting 21 of the most clinically relevant serogroups for STEC infections in humans, revealed that the STECs isolated 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 (Table S1).

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 faecal samples and one milk filter sample. The fourth isolate was isolated from a faecal sample from another farm. Both farms were using loose housing.

A higher prevalence of *stx2* positive faeces-samples was observed in the autumn compared with the spring and early summer months (Table 3). The differences between visit one (August–September) and visit five (May) ($\beta = -1.70$, $p = 0.01$) and six (June) ($\beta = -2.28$, $p = 0.02$) were statistically significant (Table S4). The highest seasonal variation in the prevalence of STEC during the sampling period was observed in milk filters between visit one (August–September) and visit five (May) ($\beta = -0.27$, $p = 0.053$) (Table S5). The prevalence of *eae* positive BTM samples was higher during visit one in August–September and visit six in June compared with the other samplings (Table 3) with a statistically significant difference between visit six and visit four ($\beta = 1.92$, $p = 0.05$) (Table S4). This was not the case for *eae* in the faecal samples, where the highest level of positive samples was observed in August to December (Table 3). However, the *eae* levels were relatively high in both faeces and BTM at visit one (August/September) (Table 3).

To summarize the results, *Campylobacter* spp. were at some point isolated from all farms and all these farms, except farm 18, had one or more positive *L. monocytogenes* samples, and six farms had one or more samples positive for STEC. A summary of these findings is shown in Table S6.

TABLE 2 Prevalence of *Campylobacter* spp. in dairy farm samples

Sampling	BTM	MF	Faeces	Teat swab	Teat milk	Total
Aug./Sept.	2/18 (11)	2/17 (12)	9/16 (56)	1/17 (6)		14/68 (21)
Nov./Dec.	1/18 (6)	1/16 (6)	15/18 (83)	2/18 (11)		19/70 (27)
Jan	0/18 (0)	0/17 (0)	11/18 (61)	3/18 (17)	1/18 (6)	15/89 (17)
Feb./Mar.	0/18 (0)	1/13 (8)	12/18 (67)	3/18 (17)	1/18 (6)	17/85 (20)
May	0/14 (0)	0/14 (0)	12/14 (86)	3/14 (21)	0/14 (0)	15/70 (21)
June	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)

Note: Prevalence of bulk tank milk- (BTM), milk filter- (MF), faeces, teat swab- and teat milk-samples positive for *Campylobacter* spp. The numbers given are positive samples/total samples (%). The samples were collected at six different time points between August 2019 and July 2020.

TABLE 3 Detection of *stx1*, *stx2* and *eae* in dairy farm samples

Sample	Visit	*stx1*	*stx2*	*eae*	*stx1/2 + eae*
Faeces	Aug./Sept.	5/15 (33)	9/15 (60)	3/15 (20)	3/15 (20)
	Nov./Dec.	6/18 (33)	8/18 (44)	4/18 (22)	4/18 (22)
	Jan.	3/18 (17)	5/18 (28)	3/18 (17)	3/18 (17)
	Feb./Mar.	1/18 (6)	5/18 (28)	2/18 (11)	1/18 (6)
	May	2/14 (14)	3/14 (21)	1/14 (7)	1/14 (7)
	June	2/15 (13)	2/15 (13)	1/15 (7)	1/15 (7)
	Total	19/98 (19)	32/98 (33)	14/98 (14)	13/98 (13)
Milk filter	Aug./Sept.	2/18 (11)	7/18 (39)	5/18 (28)	3/18 (17)
	Nov./Dec	2/18 (11)	4/18 (22)	5/18 (28)	2/18 (11)
	Jan.	1/18 (6)	3/18 (17)	4/18 (22)	2/18 (11)
	Feb./Mar.	2/16 (13)	2/16 (13)	3/16 (19)	2/16 (13)
	May	1/14 (7)	3/14 (21)	0/14 (0)	0/14 (0)
	June	1/16 (6)	6/16 (38)	7/16 (44)	3/16 (19)
	Total	9/100 (9)	25/100 (25)	24/100 (24)	12/100 (12)
Bulk tank milk	Aug./Sept.	0/18 (0)	2/18 (11)	5/18 (28)	0/18 (0)
	Nov./Dec.	0/18 (0)	1/18 (6)	2/18 (11)	0/18 (0)
	Jan.	0/18 (0)	0/18 (0)	2/18 (11)	0/18 (0)
	Feb./Mar.	1/18 (6)	2/18 (11)	1/18 (6)	1/18 (6)
	May	0/14 (0)	1/14 (7)	1/14 (7)	0/14 (0)
	June	9/14 (64)	4/14 (29)	4/14 (29)	3/14 (21)
	Total	10/100 (10)	10/100 (10)	15/100 (15)	4/100 (4)
All samples	Total	38/298 (13)	67/298 (22)	53/298 (18)	29/298 (10)

Note: Prevalence of bulk tank milk-samples, milk filter-samples and faecal-samples positive for *stx1*, *stx2* and *eae* after enrichment in mTSB at 37°C for 24 h. The numbers given are positive samples/total samples (%).

TABLE 4 Isolation of Shiga toxin-producing *Escherichia coli* from BTM, milk filters and faeces

Sampling	BTM	MF	Faeces	Total all samples
Aug./Sept.	0/18 (0)	1/18 (6)	1/15 (7)	2/51 (4)
Nov./Dec.	0/18 (0)	1/18 (6)	3[a]/18 (17)	4/54 (7)
Jan	0/18 (0)	1/18 (6)	2[a]/18 (11)	3/54 (6)
Feb./Mar.	0/18 (0)	0/16 (0)	1/18 (6)	1/52 (2)
May	0/14 (0)	1/14 (7)	2/14 (14)	3/42 (7)
June	1/14 (7)	3[a]/16 (19)	2[a]/15 (13)	6/45 (13)
Total	1/100 (1)	7/100 (7)	11/98 (11)	19/298 (6)

Note: Prevalence of Shiga toxin producing *Escherichia coli* isolates from bulk tank milk (BTM) samples, milk filter (MF) samples and faecal samples positive for *stx1*, *stx2* and *eae*. The numbers given are positive samples/total samples (%).

[a]*Escherichia coli* isolates positive for both *stx* and *eae* were isolated from three faecal samples (sampling 2, 3 and 6) and from one milk filter (sampling 6).

The prevalence of pathogens in samples from loose housing herds compared with tie-stall herds

L. monocytogenes was detected more frequently in faecal samples from loose housing herds compared with tie-stall herds (OR = 3.19, $p = 0.02$) (Table S2), with an isolation prevalence of 40% and 15% respectively (Figure 1). *L. monocytogenes* was isolated more frequently from feed samples in farms with loose housing systems compared with tie-stall farms (OR = 2.75, $p = 0.03$) (Figure 1).

L. monocytogenes was isolated from milk filters from nine out of 18 farms and there was no difference in occurrence between farms with loose stall housing systems compared 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 faecal 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. monocytogenes* in faecal samples was observed ($p < 0.01$).

There was a significant difference in the occurrence of *Campylobacter* spp. in faecal samples from farms with loose housing systems compared with tie-stall housing (OR = 3.65, $p < 0.01$) (Figure 1; Table S3). Similarly, there was a higher occurrence of *Campylobacter* spp. in teat swabs from farms with loose housing compared with

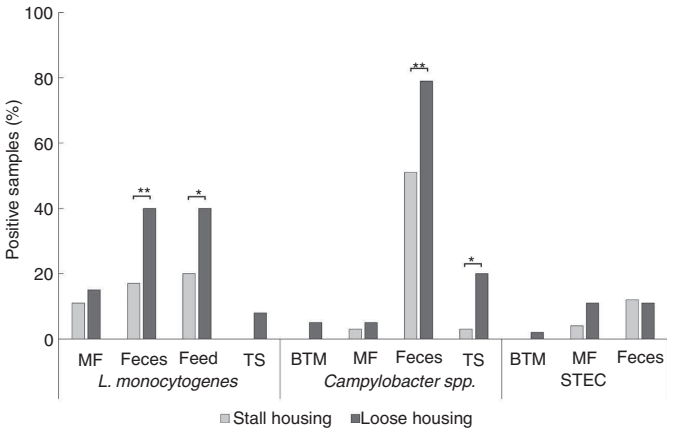

FIGURE 1 Pathogen occurrence according to housing system. Number of (%) samples positive for *Listeria monocytogenes*, *Campylobacter* spp. and Shiga toxin-producing *Escherichia coli* in loose housing versus tie-stall housing (*; $p < 0.05$, **; $p < 0.02$). MF, milk filter; BTM, bulk tank milk; TS, teat swab

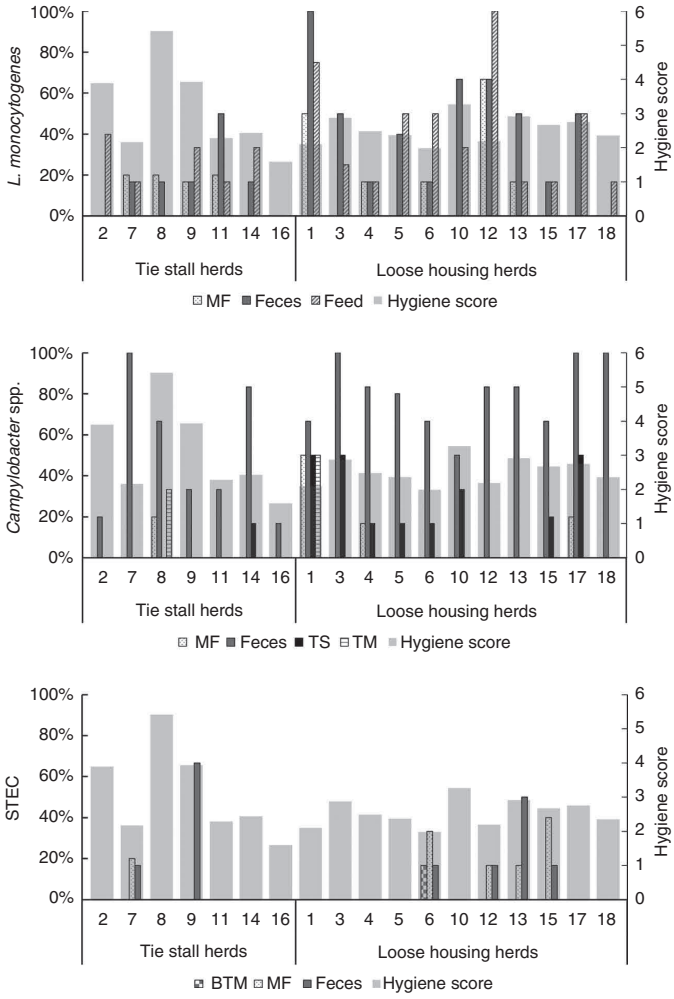

FIGURE 2 Pathogen occurrence versus dairy cattle hygiene score. % samples positive for *Listeria monocytogenes*, *Campylobacter* spp. and Shiga toxin-producing *Escherichia coli* in each herd together with average dairy cattle hygiene score. Score points (0–3) from three body zones were summarized, giving a hygiene score between zero and nine for each cow. The average dairy cattle hygiene score was calculated as the average of hygiene scores within one herd from four to six visits. No herds had an average dairy cattle hygiene score higher than six. MF = milk filter, BTM = bulk tank milk, TS = teat swab, TM = teat milk

tie-stall housing farms (OR = 9.70, $p = 0.03$). There was, however, no significant difference in the prevalence of *Campylobacter* spp. in milk filters ($p = 0.52$) or teat milk samples ($p = 0.76$) between farms having loose housing versus tie-stall systems. Neither farms with loose housing nor those with tie-stall housing showed an association between herd size and the occurrence of *Campylobacter* species in faeces samples. There was, however, an association between the isolation rate of *Campylobacter* spp. in teat swabs ($\beta = 0.03$, $p < 0.01$) and herd size regardless of housing system.

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

Seven of the 19 STECs were isolated from tie-stall herds and 12 of the isolates were from loose housing herds. STECs were isolated from faecal samples collected from four loose housing herds and from two tie-stall herds. However, four out of 11 STEC positive faecal samples (36%) came from one specific farm where the animals were tie stalled. STECs were also isolated from seven milk filters distributed over 5 out of 18 herds; one of these herds had tie-stall housing while four had loose housing. Notably, one STEC positive BTM 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 four to six sampling occasions are shown in Figure 2. We observed an association ($\beta = 0.83$, $p = 0.03$) between dairy cow hygiene score and detection of *Campylobacter* spp. in teat milk samples (Figure 2; Table S3). No association between hygiene score and detection of *L. monocytogenes* or *Campylobacter* species in BTM, milk filter, faeces, feed or teat swab was observed (Figure 2). Furthermore, no correlation was seen between dairy cow hygiene score and detection of STEC from BTM, milk filters or faeces. 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 potential 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. To generalize upon the entire Norwegian population, future studies should include additional farms from different parts of Norway.

Consumption of milk and dairy products has 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 (Artursson et al., 2018; Beckers et al., 1987; Waak et al., 2002). The detection of *L. monocytogenes* in the milk filter samples in all these studies strongly indicate that this bacterium can be 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 in accordance with *Listeria* being an environmental contaminant introduced to farm buildings through silage harvest or faecal shredding rather than being a component of the normal udder flora, which supports the 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 faecal samples. For comparison, a study from Finland reported the prevalence of *C. jejuni* in milk filter samples to be less than 1%. In the Finnish study, it was not found in BTM samples but was present in 53%

of faecal samples (Jaakkonen et al., 2019). In a 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 UPM have frequently been reported (Harrington et al., 2002; Heuvelink et al., 2009; Kenyon et al., 2020; Lehner et al., 2000; Schildt et al., 2006). 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 11 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 there is a risk of contracting campylobacteriosis after consumption of UPM.

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 STEC outbreaks are linked to consumption of contaminated beef and milk products (Sperandio & Nguyen, 2012). This study showed an STEC occurrence of 7%, 1% and 11% in milk filter, BTM and faeces samples respectively. We also observed a tendency for a higher prevalence of *stx2* genes and STEC in the faeces samples collected in August–September (visit one) compared with samples collected in May (visit five). The European Union summary report on trends and sources of zoonoses, zoonotic agents and foodborne 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 the before-mentioned Finnish study, 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 enterohaemorrhagic 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; Gould et al., 2013; Hughes et al., 2006). Since new STEC variants are continuously emerging, all serotypes should be considered as potential pathogens (Bielaszewska et al., 2011; Rasko et al., 2011). Notably, even the presence of low levels of STEC 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 & Nguyen, 2012).

The primer-panel used for geno-serotyping was described by Sánchez et al. (2015), and was 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 prevalences of 7%–15% for BTM samples and 40%–50% for milk filter samples (Jaakkonen et al., 2019; Van Kessel et al., 2011). 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 25% of the milk filter samples in this study were positive for *eae*, indicating a high likelihood for the presence of Intimin positive *E. coli* isolates (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 STEC in raw milk. The lack of significant association between the *eae* content in faeces and in BTM observed during the year may be due to the size of the study, and larger studies are needed to address if detection of *eae* in BTM coincides with a high detection rate of *eae* in faeces. Summer and autumn season have been shown to be significant risk factors for human STEC infections (European Centre for Disease Prevention and Control, 2021; Mughini-Gras

et al., 2018), and cattle have been shown to excrete more in warm temperatures (Venegas-Vargas et al., 2016). The current study indicates a similar pattern for dairy cattle in Norway, as *stx2* were significantly more prevalent in faeces in the autumn compared to spring and early summer, and *eae* in BTM were significantly more prevalent in summer and early autumn compared to the other samplings. Although the findings of this study indicate a higher prevalence of STEC shedding during summer and autumn season further studies are needed to conclude.

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 faeces and teat swabs and *L. monocytogenes* in faeces and feed was higher in loose housed herds compared with 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 with tie-stalled, which confers more animal-to-animal interactions and increased faecal contamination of the environment.

The hygiene of dairy cows can be used as an indicator of animal welfare and the quality of the farm facilities (Hultgren & Bergsten, 2001; Welfare Quality Consortium, 2009) and poor hygiene are associated with an increased occurrence of mastitis and high somatic cell counts (Cook & Reinemann, 2006; Schreiner & Ruegg, 2003). Poor udder hygiene has been associated with dirty environment (Devries et al., 2012) and pathogens are shown to be transmitted to milk via dirt from the udder (Vissers et al., 2007). Our study indicates an association between cow hygiene and detection of *Campylobacter* spp. in teat milk samples. The cow hygiene is likely to depend on the state of the surrounding environment during the different seasons. An Italian study reports that cows were significantly dirtier in December, January and February compared with April and October and they suggested that difficulties in keeping the bedding dry during the rainy season resulted in an increased amount of manure on legs, flanks, and udders (Zucali et al., 2011).

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 with August/September, May, and June (22%, 21% and 20% respectively). Notably, only January compared with 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 (Castro et al., 2018). 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). 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) and some studies found higher *L. monocytogenes* levels during the summer season (Dalzini et al., 2016; Hutchison et al., 2005). 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. Dairy cattle grazing practices in Norway varies across climatic zones, and the farms included in this study were located in a typical inland climate, characterized by a relatively short grazing season. In this region, silage is provided both during housing- and grazing seasons in combination with concentrates to compensate for feed intake, feed quality and nutritional requirements according to the individual milk production. The silage is generally stored in sealed bales, silos or in silage pits until use. Associations between feeding practices, silage storage methods, feed composition and *L. monocytogenes* contamination were not part of the current investigation.

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, such as *Campylobacter* spp. and STEC, 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

No conflict of interest declared.

DATA AVAILABILITY STATEMENT

The datasets supporting the conclusions of this article are included within the article and its additional files.

ORCID

Toril Lindbäck https://orcid.org/0000-0002-5245-1087

REFERENCES

Alegbeleye, O.O., Guimarães, J.T., Cruz, A.G. & Sant'Ana, A.S. (2018) Hazards of a 'healthy' trend? An appraisal of the risks of raw milk consumption and the potential of novel treatment technologies to serve as alternatives to pasteurization. *Trends in Food Science and Technology*, 82, 148–166.

An, J.U., Ho, H., Kim, J., Kim, W.H., Kim, J., Lee, S. et al. (2018) Dairy cattle, a potential reservoir of human campylobacteriosis: epidemiological and molecular characterization of *Campylobacter jejuni* from cattle farms. *Frontiers in Microbiology*, 9, 3136.

Artursson, K., Schelin, J., Thisted Lambertz, S., Hansson, I. & Olsson Engvall, E. (2018) Foodborne pathogens in unpasteurized milk in Sweden. *International Journal of Food Microbiology*, 284, 120–127.

Beckers, H.J., Soentoro, P.S.S. & Delgou-van Asch, E.H.M. (1987) The occurrence of *Listeria monocytogenes* in soft cheeses and raw milk and its resistance to heat. *International Journal of Food Microbiology*, 4, 249–256.

Bielaszewska, M., Mellmann, A., Zhang, W., Köck, R., Fruth, A., Bauwens, A. et al. (2011) Characterisation of the *Escherichia coli* strain associated with an outbreak of haemolytic uraemic syndrome in Germany, 2011: a microbiological study. *The Lancet Infectious Diseases*, 11, 671–676.

Borucki, M.K., Gay, C.C., Reynolds, J., McElwain, K.L., Kim, S.H., Call, D.R. et al. (2005) Genetic diversity of *Listeria monocytogenes* strains from a high-prevalence dairy farm. *Applied and Environmental Microbiology*, 71, 5893–5899.

Brooks, J.T., Sowers, E.G., Wells, J.G., Greene, K.D., Griffin, P.M., Hoekstra, R.M. et al. (2005) Non-O157 Shiga toxin–producing *Escherichia coli* infections in the United States, 1983–2002. *The Journal of Infectious Diseases*, 192, 1422–1429.

Castro, H., Jaakkonen, A., Hakkinen, M., Korkeala, H. & Lindström, M. (2018) Occurrence, persistence, and contamination routes of *Listeria monocytogenes* genotypes on three finnish dairy cattle farms: a longitudinal study. *Applied and Environmental Microbiology*, 84, e02000–e02017.

Castro, H., Ruusunen, M. & Lindström, M. (2017) Occurrence and growth of *Listeria monocytogenes* in packaged raw milk. *International Journal of Food Microbiology*, 261, 1–10.

Chlebicz, A. & Śliżewska, K. (2018) Campylobacteriosis, salmonellosis, yersiniosis, and listeriosis as zoonotic foodborne diseases: a review. *International Journal of Environmental Research and Public Health*, 15, 863.

Claeys, W.L., Cardoen, S., Daube, G., De Block, J., Dewettinck, K., Dierick, K. et al. (2013) Raw or heated cow milk consumption: review of risks and benefits. *Journal of Food Control*, 31, 251–262.

Cogato, A., Brščić, M., Guo, H., Marinello, F. & Pezzuolo, A. (2021) Challenges and tendencies of automatic milking systems (AMS): A 20-years systematic review of literature and patents. *Animals*, 11, 356.

Cook, N.B. (2002) *The influence of barn design on dairy cow hygiene, lameness and udder health*. Madison, Wisconsin, USA: American Association of Bovine Practitioners thirty-fifth annual conference.

Cook, N.B. & Reinemann, D. (2006) *A tool box for assessing cow, udder and teat hygiene*. Madison, Wisconsin, USA: Annual meeting of the National Mastitis Council.

Crotta, M., Paterlini, F., Rizzi, R. & 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. *Journal of Dairy Science*, 99, 1029–1038.

Dalzini, E., Bernini, V., Bertasi, B., Daminelli, P., Losio, M.N. & 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. & Lafarge, V. (2001) Implication of milk and milk products in food-borne diseases in France and in different industrialised countries. *International Journal of Food Microbiology*, 67, 1–17.

de Koning, K., Slaghuis, B. & van der Vorst, Y. (2003) Robotic milking and milk quality. Effects on bacterial counts - somatic cell counts - freezing point and free fatty acids. *Italian Journal of Animal Science*, 2, 291–299.

DebRoy, C., Fratamico, P.M., Yan, X., Baranzoni, G., Liu, Y., Needleman, D.S. et al. (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.

Devries, T.J., Aarnoudse, M.G., Barkema, H.W., Leslie, K.E. & von Keyserlingk, M.A. (2012) Associations of dairy cow behavior, barn hygiene, cow hygiene, and risk of elevated somatic cell count. *Journal of Dairy Science*, 95, 5730–5739.

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

Epps, S., Harvey, R., Hume, M., Phillips, T., Anderson, R. & Nisbet, D. (2013) Foodborne Campylobacter: Infections, Metabolism, Pathogenesis and Reservoirs. *International Journal of Environmental Research and Public Health*, 10, 6292–6304.

European Centre for Disease Prevention and Control (2021). Shiga toxin-producing *Escherichia coli* (STEC) infection. Annual epidemiological report for 2019. Stockholm.

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 Journal*, 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 Journal*, 16, e05500.

Gaya, P., Sanchez, J., Medina, M. & Nuñez, M. (1998) Incidence of *Listeria monocytogenes* and other Listeria species in raw milk produced in Spain. *Food Microbiology*, 15, 551–555.

Gould, L.H., Mody, R.K., Ong, K.L., Clogher, P., Cronquist, A.B., Garman, K.N. et al. (2013) Increased recognition of non-O157 Shiga toxin–producing *Escherichia coli* infections in the United States during 2000–2010: epidemiologic features and comparison with *E. coli* O157 infections. *Foodborne Pathogens and Disease*, 10, 453–460.

Hansen, B.G., Herje, H.O. & Höva, J. (2019) Profitability on dairy farms with automatic milking systems compared to farms with

conventional milking systems. *Int Food Agribusiness Manag*, 22, 215–228.

Harrington, P., Archer, J., Davis, J.P., Croft, D.R. & Varma, J. K. (2002) *Outbreak of Campylobacter jejuni infections associated with drinking unpasteurized milk procured through a cow-leasing program--Wisconsin, 2001*. Morbidity and Mortality Weekly Report No. 0149-2195. Atlanta: Centers for Disease Control Prevention, pp. 548–549.

Hassan, L., Mohammed, H.O. & McDonough, P.L. (2001) Farm-management and milking practices associated with the presence of *Listeria monocytogenes* in New York state dairy herds. *Preventive Veterinary Medicine*, 51, 63–73.

Heuvelink, A.E., van Heerwaarden, C., Zwartkruis-Nahuis, A., Tilburg, J.J.H.C., Bos, M.H., Heilmann, F.G.C. et al. (2009) Two outbreaks of campylobacteriosis associated with the consumption of raw cows' milk. *International Journal of Food Microbiology*, 134, 70–74.

Hovinen, M. & Pyörälä, S. (2011) Invited review: Udder health of dairy cows in automatic milking. *Journal of Dairy Science*, 94, 547–562.

Hovinen, M., Rasmussen, M.D. & Pyörälä, S. (2009) Udder health of cows changing from tie stalls or free stalls with conventional milking to free stalls with either conventional or automatic milking. *Journal of Dairy Science*, 92, 3696–3703.

Hughes, J.M., Wilson, M.E., Johnson, K.E., Thorpe, C.M. & Sears, C.L. (2006) The emerging clinical importance of non-O157 Shiga toxin—producing *Escherichia coli*. *Clinical Infectious Diseases*, 43, 1587–1595.

Hultgren, J. & Bergsten, C. (2001) Effects of a rubber-slatted flooring system on cleanliness and foot health in tied dairy cows. *Preventive Veterinary Medicine*, 52, 75–89.

Hutchison, M.L., Walters, L.D., Avery, S.M., Munro, F. & Moore, A. (2005) Analyses of livestock production, waste storage, and pathogen levels and prevalences in farm manures. *Applied and Environmental Microbiology*, 71, 1231–1236.

Jaakkonen, A., Castro, H., Hallanvuo, S., Ranta, J., Rossi, M., Isidro, J. et al. (2019) Longitudinal study of shiga toxin-producing *Escherichia coli* and *Campylobacter jejuni* on Finnish Dairy farms and in raw milk. *Applied and Environmental Microbiology*, 85, e02910–e02918.

Jacobs, J.A. & Siegford, J.M. (2012) Invited review: The impact of automatic milking systems on dairy cow management, behavior, health, and welfare. *Journal of Dairy Science*, 95, 2227–2247.

Jørgensen, H.J., Mørk, T. & Rørvik, L.M. (2005) The occurrence of *Staphylococcus aureus* on a farm with small-scale production of raw milk cheese. *Journal of Dairy Science*, 88, 3810–3817.

Kenyon, J., Inns, T., Aird, H., Swift, C., Astbury, J., Forester, E. et al. (2020) Campylobacter outbreak associated with raw drinking milk, North West England, 2016. *Epidemiology and Infection*, 148, e13.

Klungel, G., Slaghuis, B. & Hogeveen, H. (2000) The effect of the introduction of automatic milking systems on milk quality. *Journal of Dairy Science*, 83, 1998–2003.

Lahti, E., Rehn, M., Ockborn, G., Hansson, I., Ågren, J., Engvall, E.O. et al. (2017) Outbreak of campylobacteriosis following a dairy farm visit: confirmation by genotyping. *Foodborne Pathogens and Disease*, 14, 326–332.

Langer, A.J., Ayers, T., Grass, J., Lynch, M., Angulo, F.J. & Mahon, B.E. (2012) Nonpasteurized dairy products, disease outbreaks, and state laws-United States, 1993-2006. *Emerging Infectious Diseases*, 18, 385–391.

Lehner, A., Schneck, C., Feierl, G., Pless, P., Deutz, A., Brandl, E. et al. (2000) Epidemiologic application of pulsed-field gel electrophoresis to an outbreak of *Campylobacter jejuni* in an Austrian youth centre. *Epidemiology and Infection*, 125, 13–16.

Łoś, J.M., Łoś, M. & Węgrzyn, G. (2011) Bacteriophages carrying Shiga toxin genes: genomic variations, detection and potential treatment of pathogenic bacteria. *Future Microbiology*, 6, 909–924.

Lucey, J.A. (2015) Raw milk consumption: risks and benefits. *Nutrition Today*, 50, 189–193.

Ludwig, J.B., Shi, X., Shridhar, P.B., Roberts, E.L., DebRoy, C., Phebus, R.K. et al. (2020) Multiplex PCR assays for the detection of one hundred and thirty seven serogroups of Shiga toxin-producing *Escherichia coli* associated with cattle. *Frontiers in Cellular and Infection Microbiology*, 10, 378.

Lundén, J., Tolvanen, R. & Korkeala, H. (2004) Human listeriosis outbreaks linked to dairy products in Europe. *Journal of Dairy Science*, 87, e6–e12.

Monday, S.R., Beisaw, A. & Feng, P.C.H. (2007) Identification of Shiga toxigenic *Escherichia coli* seropathotypes A and B by multiplex PCR. *Molecular and Cellular Probes*, 21, 308–311.

Mughini-Gras, L., van Pelt, W., van der Voort, M., Heck, M., Friesema, I. & Franz, E. (2018) Attribution of human infections with Shiga toxin-producing *Escherichia coli* (STEC) to livestock sources and identification of source-specific risk factors, The Netherlands (2010–2014). *Zoonoses and Public Health*, 65, e8–e22.

Nightingale, K.K., Fortes, E.D., Ho, A.J., Schukken, Y.H., Grohn, Y.T. & Wiedmann, M. (2005) Evaluation of farm management practices as risk factors for clinical listeriosis and fecal shedding of *Listeria monocytogenes* in ruminants. *Journal of the American Veterinary Medical Association*, 227, 1808–1814.

Nørstebø, H., Rachah, A., Dalen, G., Rønningen, O., Whist, A.C. & Reksen, O. (2018) Milk-flow data collected routinely in an automatic milking system: an alternative to milking-time testing in the management of teat-end condition? *Acta Veterinaria Scandinavica*, 60, 2–9.

Norwegian Veterinary Institute (2019) The surveillance programmes for Salmonella in live animals, eggs and meat in Norway 2019, annual report. p. 3.

Rasko, D.A., Webster, D.R., Sahl, J.W., Bashir, A., Boisen, N., Scheutz, F. et al. (2011) Origins of the *E. coli* strain causing an outbreak of hemolytic–uremic syndrome in Germany. *The New England Journal of Medicine*, 365, 709–717.

Rasmussen, M.D., Bjerring, M., Justesen, P. & Jepsen, L. (2002) Milk quality on Danish farms with automatic milking systems. *Journal of Dairy Science*, 85, 2869–2878.

Rasmussen, M.D., Blom, J.Y., Nielsen, L.A.H. & Justesen, P. (2001) Udder health of cows milked automatically. *Livestock Production Science*, 72, 147–156.

Ricci, A., Allende, A., Bolton, D., Chemaly, M., Davies, R., Fernández Escámez, P.S. et al. (2018) *Listeria monocytogenes* contamination of ready-to-eat foods and the risk for human health in the EU. *EFSA Journal*, 16, e05134-n/a.

Riley, L.W., Remis, R.S., Helgerson, S.D., McGee, H.B., Wells, J.G., Davis, B.R. et al. (1983) Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. *The New England Journal of Medicine*, 308, 681–685.

Roberts, A.J. & Wiedmann, M. (2003) Pathogen, host and environmental factors contributing to the pathogenesis of listeriosis. *Cellular and Molecular Life Sciences*, 60, 904–918.

Sánchez, S., Llorente, M.T., Echeita, M.A. & Herrera-León, S. (2015) Development of three multiplex PCR assays targeting the 21 most clinically relevant serogroups associated with Shiga toxin-producing *E. coli* infection in humans. *PLoS One*, 10, e0117660.

Sapountzis, P., Segura, A., Desvaux, M. & Forano, E. (2020) An overview of the elusive passenger in the gastrointestinal tract of cattle: the shiga toxin producing *Escherichia coli*. *Microorganisms (Basel)*, 8, 877.

Schildt, M., Savolainen, S. & Hänninen, M.L. (2006) Long-lasting *Campylobacter jejuni* contamination of milk associated with gastrointestinal illness in a farming family. *Epidemiology and Infection*, 134, 401–405.

Schmidt, M.A. (2010) LEEways: tales of EPEC, ATEC and EHEC. *Cellular Microbiology*, 12, 1544–1552.

Schreiner, D.A. & Ruegg, P.L. (2003) Relationship between udder and leg hygiene scores and subclinical mastitis. *Journal of Dairy Science*, 86, 3460–3465.

Sekse, C., Solberg, A., Petersen, A., Rudi, K. & Wasteson, Y. (2005) Detection and quantification of Shiga toxin-encoding genes in sheep faeces by real-time PCR. *Molecular and Cellular Probes*, 19, 363–370.

Sonnier, J.L., Karns, J.S., Lombard, J.E., Kopral, C.A., Haley, B.J., Kim, S.W. et al. (2018) Prevalence of *Salmonella enterica*, *Listeria monocytogenes*, and pathogenic *Escherichia coli* in bulk tank milk and milk filters from US dairy operations in the National Animal Health Monitoring System Dairy 2014 study. *Journal of Dairy Science*, 101, 1943–1956.

Sperandio, V. & Nguyen, Y. (2012) Enterohemorrhagic *E. coli* (EHEC) pathogenesis. *Frontiers in Cellular and Infection Microbiology*, 2, 90.

Statens Serum Institut (2018) Campylobacter infections, 2016–2017. Annual reports on disease incidence, Denmark.

Steele, J.H. (2000) History, trends, and extent of pasteurization. *Journal of the American Veterinary Medical Association*, 217, 175–178.

van der Vorst, Y. and Hogeveen, H. (2000) *Automatic milking systems and milk quality in The Netherlands*. Robotic milking: Proceedings of the International Symposium held in Lelystad, The Netherlands, 17–19 August, 2000. Wageningen Pers. pp.73-82.

Van der Vorst, Y. & Ouweltjes, W. (2003) *Milk quality and automatic milking; a risk inventory*. Lelystad, The Netherlands: Praktijkonderzoek Veehouderij.

Van Kessel, J.A.S., Karns, J.S., Lombard, J.E. & Kopral, C.A. (2011) Prevalence of *Salmonella enterica*, *Listeria monocytogenes*, and *Escherichia coli* Virulence Factors in Bulk Tank Milk and In-Line Filters from U.S. Dairies. *Journal of Food Protection*, 74, 759–768.

Venegas-Vargas, C., Henderson, S., Khare, A., Mosci, R.E., Lehnert, J.D., Singh, P. et al. (2016) Factors associated with shiga toxin-producing *Escherichia coli* shedding by dairy and beef cattle. *Applied and Environmental Microbiology*, 82, 5049–5056.

Vissers, M.M., Driehuis, F., Te Giffel, M.C., De Jong, P. & Lankveld, J.M. (2007) Short communication: Quantification of the transmission of microorganisms to milk via dirt attached to the exterior of teats. *Journal of Dairy Science*, 90, 3579–3582.

Waak, E., Tham, W. & Danielsson-Tham, M.L. (2002) Prevalence and fingerprinting of *Listeria monocytogenes* strains isolated from raw whole milk in farm bulk tanks and in dairy plant receiving tanks. *Applied and Environmental Microbiology*, 68, 3366–3370.

Welfare Quality Consortium. (2009) *Welfare quality assessment protocol for cattle*. Lelystad, the Netherlands: Anon.

Yoshida, T., Kato, Y., Sato, M. & Hirai, K. (1998) Sources and routes of contamination of raw milk with *Listeria monocytogenes* and its control. *The Journal of Veterinary Medical Science*, 60, 1165–1168.

Zucali, M., Bava, L., Tamburini, A., Brasca, M., Vanoni, L. & Sandrucci, A. (2011) Effects of season, milking routine and cow cleanliness on bacterial and somatic cell counts of bulk tank milk. *The Journal of Dairy Research*, 78, 436–441.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Idland, L., Granquist, E.G., Aspholm, M. & Lindbäck, T. (2022) The prevalence 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. *Journal of Applied Microbiology*, 132, 3959–3972. Available from: https://doi.org/10.1111/jam.15512

Table S1 Primers used in the study

Primer name	Primer sequence (5′-3′)	Reference
Stx2F	GCGTTTTGACCATCTTCGT	Muniesa et al. 1998
Stx2R	ACAGGAGCAGTTTCAGACAG	Muniesa et al. 1998
Stx1F (stx1M16625f)	GATAGTGGCTCAGGGGATAAT	Sekse et al. 2005
Stx1R	GCCGAAAACGTAAAGCTTCAG	This study
EaeF	GTGGCGAATACTGGCGAGACT	Paton et al. 1998
EaeR	CTTGTGCGCTTTGGCTTC	This study
O5F	CTTATCCGATTAATGGCTTC	Sánchez et al. 2015
O5R	TAGTCGATTTGCTTTTATGG	Sánchez et al. 2015
O91F	TTTTCTGGAATGCTTGATGA	Sánchez et al. 2015
O91R	ATAATTTTACGCCGTGTTTG	Sánchez et al. 2015
O26F	ACTCTTGCTTCGCCTGTT	Monday et al. 2007
O26R	CAGCGATACTTTGAACCTTAT	Monday et al. 2007
O103F	TATCCTTCATAGCCTGTTGTT	Monday et al. 2007
O103R	TTATAATAGTAATAAGCCAGACACC	Sánchez et al. 2015
O145F	TTGAGCACTTATCACAAGAGATT	Monday et al. 2007
O145R	GATTGAATAGCTGAAGTCATACTAAC	Monday et al. 2007
O121F	GTAGCGAAAGGTTAGACTGG	Monday et al. 2007
O121R	ATGGGAAAGCTGATACTGC	Monday et al. 2007
O111F	GTTGCGAGGAATAATTCTTCA	Monday et al. 2007
O111R	CCATAGATATTGCATAAAGGC	Monday et al. 2007
O55F	ATCGCAATTGCAATAAACTC	Sánchez et al. 2015
O55R	CCCAACTCTAGTAGATAAAAGCC	Sánchez et al. 2015
O128F	TTTCGATCGTCTTGTTCAGG	Sánchez et al. 2015
O128R	CAATGGGCAATTAACACAGAG	Sánchez et al. 2015
O113F	TAACGGGATTAGAAGTGGAT	Sánchez et al. 2015
O113R	ATATAAGGCAGAAATGAGAGG	Sánchez et al. 2015
O146F	ATCAGTTCATGGGTTGTATTC	Sánchez et al. 2015
O146R	AGGAACATGGATGAAAGAAG	Sánchez et al. 2015
O45F	GACTTTCGTTGCGTTGTG	Sánchez et al. 2015
O45R	CTGCAAGTGTAGCGAAAAC	Sánchez et al. 2015
O177F	TCGGTGTTTGAAGGGGAAG	Sánchez et al. 2015

O177R	GTCCATGCATATGCCGTTC	Sánchez et al. 2015
O157F	CTCAATTTATAAAAAAGACGCTC	Sánchez et al. 2015
O157R	TCCAAATATTAACGACTTCACTAC	Sánchez et al. 2015
O15F	GCGTTGCCTACTTACTTATTATC	Sánchez et al. 2015
O15R	ATGCAAGTCCAGCCAAAC	Sánchez et al. 2015
O104F	CGGTGTATTAAGAAGTGTTGTC	Sánchez et al. 2015
O104R	ATACTCCCCATAGAAACGC	Sánchez et al. 2015
O118F	TGGAGAACAGATAGCAAGAGG	Sánchez et al. 2015
O118R	TATCCGACAAACACGAACC	Sánchez et al. 2015
O123F	GAAAGAACAGAATCAGACTATGC	Sánchez et al. 2015
O123R	TGTGCTAGCGCTAAAGGAC	Sánchez et al. 2015
O165F	AACTGTTTATCCGAAGTGGTAG	Sánchez et al. 2015
O165R	CACGCTTTAACGCATACAG	Sánchez et al. 2015
O172F	ATTGGGTAGCCTCAGTAAAG	Sánchez et al. 2015
O172R	CAGTCCAAACAGTGACAGTATC	Sánchez et al. 2015

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

Table S6 Number positive samples from the individual dairy farms

Farm	*L. monocytogenes*	*Campylobacter* spp.	STEC
1*	8/19	7/15	0/11
2	2/26	1/21	0/14
3*	4/20	6/16	0/12
4*	3/34	8/28	0/17
5*	5/33	5/27	0/17
6*	5/34	5/28	4/18
7	3/33	6/27	2/17
8	2/31	6/25	0/18
9	4/34	2/28	4/18
10*	6/33	5/27	0/18
11	5/33	2/27	0/18
12*	15/34	5/28	2/18
13*	6/34	5/28	4/18
14	3/34	6/28	0/18
15*	3/32	5/26	3/17
16	0/34	1/28	0/18
17*	4/24	8/20	0/13
18*	1/34	6/28	0/18
Total	79/556	89/455	19/298

Number of dairy farm samples positive for *L. monocytogenes*, *Campylobacter* spp. and STEC regardless of sample type. *Loose housed herds

Paper II

AMERICAN SOCIETY FOR MICROBIOLOGY | Applied and Environmental Microbiology®

FOOD MICROBIOLOGY

Whole-Genome Sequencing Analysis of *Listeria monocytogenes* from Rural, Urban, and Farm Environments in Norway: Genetic Diversity, Persistence, and Relation to Clinical and Food Isolates

Annette Fagerlund,[a] Lene Idland,[b] Even Heir,[a] Trond Møretrø,[a] Marina Aspholm,[b] Toril Lindbäck,[b] Solveig Langsrud[a]

[a]Nofima, Norwegian Institute of Food, Fisheries and Aquaculture Research, Ås, Norway
[b]Department of Paraclinical Sciences, Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Ås, Norway

Annette Fagerlund and Lene Idland contributed equally to this article. Author order was determined by mutual agreement.

ABSTRACT *Listeria monocytogenes* is a ubiquitous environmental bacterium associated with a wide variety of natural and human-made environments, such as soil, vegetation, livestock, food processing environments, and urban areas. It is also among the deadliest foodborne pathogens, and knowledge about its presence and diversity in potential sources is crucial to effectively track and control it in the food chain. Isolation of *L. monocytogenes* from various rural and urban environments showed higher prevalence in agricultural and urban developments than in forest or mountain areas, and that detection was positively associated with rainfall. Whole-genome sequencing (WGS) was performed for the collected isolates and for *L. monocytogenes* from Norwegian dairy farms and slugs (218 isolates in total). The data were compared to available data sets from clinical and food-associated sources in Norway collected within the last decade. Multiple examples of clusters of isolates with 0 to 8 whole-genome multilocus sequence typing (wgMLST) allelic differences were collected over time in the same location, demonstrating persistence of *L. monocytogenes* in natural, urban, and farm environments. Furthermore, several clusters with 6 to 20 wgMLST allelic differences containing isolates collected across different locations, times, and habitats were identified, including nine clusters harboring clinical isolates. The most ubiquitous clones found in soil and other natural and animal ecosystems (CC91, CC11, and CC37) were distinct from clones predominating among both clinical (CC7, CC121, and CC1) and food (CC9, CC121, CC7, and CC8) isolates. The analyses indicated that ST91 was more prevalent in Norway than other countries and revealed a high proportion of the hypovirulent ST121 among Norwegian clinical cases.

IMPORTANCE *Listeria monocytogenes* is a deadly foodborne pathogen that is widespread in the environment. For effective management, both public health authorities and food producers need reliable tools for source tracking, surveillance, and risk assessment. For this, whole-genome sequencing (WGS) is regarded as the present and future gold standard. In the current study, we use WGS to show that *L. monocytogenes* can persist for months and years in natural, urban, and dairy farm environments. Notably, clusters of almost identical isolates, with genetic distances within the thresholds often suggested for defining an outbreak cluster, can be collected from geographically and temporally unrelated sources. The work highlights the need for a greater knowledge of the genetic relationships between clinical isolates and isolates of *L. monocytogenes* from a wide range of environments, including natural, urban, agricultural, livestock, food production, and food processing environments, to correctly interpret and use results from WGS analyses.

KEYWORDS *Listeria monocytogenes*, whole-genome sequencing, WGS, source tracking, persistence, molecular epidemiology, environmental pathogens, dairy farms

Editor Edward G. Dudley, The Pennsylvania State University

Address correspondence to Annette Fagerlund, annette.fagerlund@nofima.no.

The authors declare no conflict of interest.

Received 5 November 2021
Accepted 26 January 2022
Accepted manuscript posted online 2 February 2022
Published 22 March 2022

Listeria monocytogenes is a bacterial pathogen responsible for the life-threatening disease listeriosis. The most common cause of listeriosis is considered to be ingestion of food contaminated by *L. monocytogenes* from unclean food production equipment (1, 2). *L. monocytogenes* is a ubiquitous environmental bacterium that has been associated with a wide variety of environments, such as rivers, soil, vegetation, wild and domesticated animals, food processing environments, and urban areas (3, 4). Consequently, a total absence of *L. monocytogenes* in non-heat-treated foods is difficult, perhaps impossible, to achieve. The literature is, however, not fully consistent about the main habitats of *L. monocytogenes* and the factors affecting its occurrence and spread to humans. It is therefore of importance to increase the understanding of the relationship between *L. monocytogenes* in natural and animal reservoirs, food processing environments, and human clinical disease.

The occurrence of *L. monocytogenes* in soil varies widely, from 0.7% to 45%, depending on the geographic area, season, and humidity (4–6). In comparative investigations, higher frequencies of *L. monocytogenes* have been found after rain, flooding, and irrigation events (7, 8). Several studies have reported high incidence of *L. monocytogenes* in water from rivers and lakes, with frequencies from 10 to 62% of the samples depending on the area and detection method (9–13). A link has been found between the proximity to upstream dairy farms and cropped land and the presence of *L. monocytogenes* in river water (10, 12). An explanation for this could be high frequencies of *L. monocytogenes* in feces from farm animals, e.g., cattle, ducks, and sheep, leaking into surrounding soil and water (9, 14, 15). Dairy farms are, for example, known to hold an *L. monocytogenes* reservoir, and prevalences in environmental samples of 11 to 24% have been reported (6, 15–17). However, *L. monocytogenes* is not particularly linked to farm animals and is frequently found in other animals and birds, such as game and urban birds, boars, garden slugs, and rodents (9, 18–21). An association between dense populations of humans and occurrence of *L. monocytogenes* in the environment has been reported. A U.S. study showed that 4.4% of samples from urban or residential areas contained *L. monocytogenes*, while the pathogen was less frequently found in samples from forests and mountains (1.3%) (22).

L. monocytogenes comprises four separate deep-branching lineages, which, from an evolutionary viewpoint, could be considered separate species (23). These are further subdivided by multilocus sequence typing (MLST) into sequence types (STs) and clonal complexes (CCs or clones). The lineage I clones CC1, CC2, CC4, and CC6 are reported to be associated with human disease, while lineage II clones CC9 and CC121 are strongly associated with food and food processing environments (24–28). While many studies have examined the molecular genotypes of *L. monocytogenes* isolates found in food, food processing environments, and clinical disease, much less is known about the diversity present in other environments. In the few published studies, the clonal diversity in environmental samples from soil and water appears to be very high, sometimes dominated by CCs associated with disease (CC1 and CC4), although other dominating clones (e.g., CC37) have also been observed (5, 13, 29). Several clones are also found in wild animals, e.g., CC7 and CC37, found in moose, boars, slugs, and game birds (18, 21, 30, 31). In environmental samples from dairy/cattle farms in Finland and Latvia, the lineage II clones CC11 (ST451), CC14, CC18, CC20, CC37, and CC91 were most predominant, while lineage I clones were rare (6, 32). Although there are some exceptions, e.g., CC1 being predominant in slugs collected in garden and farm environments in Norway (21), the majority of the clones identified in natural and farm environments do not seem to belong to CCs dominant among European food and clinical isolates.

Many studies have described persistence over time for *L. monocytogenes* clones in food processing facilities (2, 33, 34) and in individual cattle herds or farm environments (15, 35, 36). Whether *L. monocytogenes* can persist over long periods of time also in rural, urban, or agricultural environments has rarely been investigated. Studies of genetic relationships between *L. monocytogenes* isolates from natural and animal reservoirs and isolates from food and clinical sources are scarce. High-resolution molecular

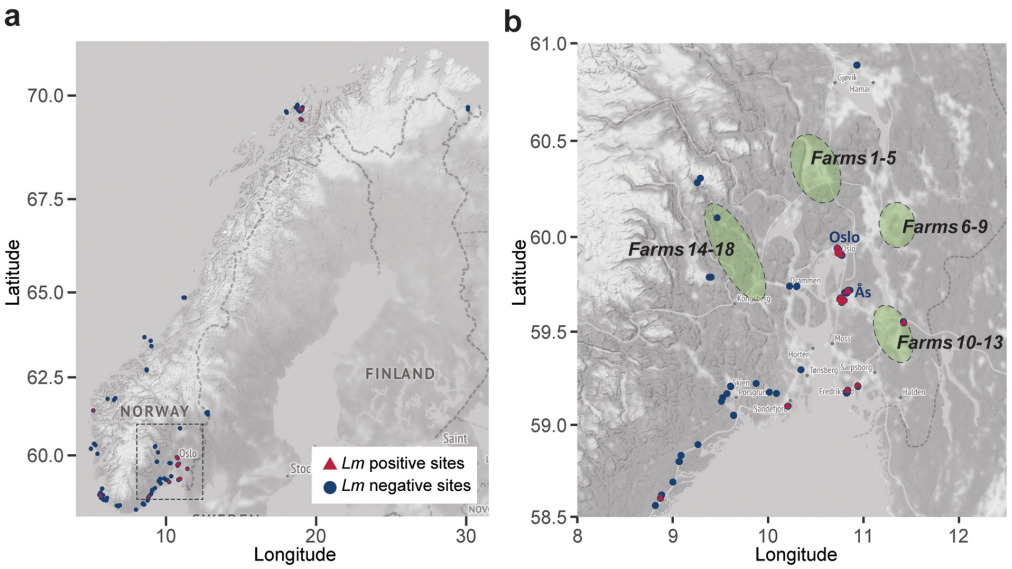

FIG 1 Maps showing the geographic location of sampling sites. (a) The location of sampling sites in rural and urban environments in Norway, with red triangles representing *L. monocytogenes*-positive samples and blue circles negative sampling points. The area outlined by the dashed square in panel a is the area shown in panel b. The green-shaded areas in panel b show the geographical origins of the dairy cattle farms sampled for *L. monocytogenes* in Idland et al. (16). The maps were plotted using the R package ggmap (66) using data from OpenStreetMap under the Open Database License (ODbL).

fingerprinting based on whole-genome sequencing (WGS) technology has revolutionized the ability to detect outbreaks and the presence of persistent strains (37). However, few studies have carried out WGS analyses of *L. monocytogenes* isolates collected from non-food-associated locations over the span of months and years. The present study aimed to use WGS to investigate the diversity and genetic relationships between *L. monocytogenes* isolates from rural, agricultural, and urban environments in Norway and to compare these with available data sets containing genomes of *L. monocytogenes* from human clinical and food-associated sources in Norway collected within the last decade.

RESULTS

Prevalence of *L. monocytogenes* in rural and urban environments in Norway. A total of 618 distinct environmental sites from rural and urban environments were sampled for *L. monocytogenes* between April 2016 and April 2020. The overall sampling scheme was designed to obtain an overview of the presence of *L. monocytogenes* in various habitats, and samples were collected from several geographical regions in Norway (Fig. 1a). To study potential persistence of *L. monocytogenes* clones over time, some sites were sampled more than once. At the onset of the study, we hypothesized that the presence of *L. monocytogenes* would be more strongly associated with farm animals, agricultural activity, and urban areas than with natural forests and other wildlands (22). During the first sampling occasion, 10% of sample sites were positive for *L. monocytogenes* (Table 1). In addition, 13 samples of commercial bags of plant soil or compost were negative for *L. monocytogenes*. In concordance with our hypothesis, the prevalence of *L. monocytogenes* was significantly higher in urban areas and in areas associated with agriculture and livestock (agricultural fields, grazelands, and animal paths) than in forest/mountain areas and on footpaths ($P < 0.02$ by Fisher's exact test). Sampling locations classified as footpaths were generally from nonurban areas, such as woods or other areas used for hiking. While 14% of urban areas were positive for

TABLE 1 Prevalence of *L. monocytogenes* in rural and urban environments

Habitat or sampling area	No. of collected isolates	Prevalence of *L. monocytogenes* (%)
Grazeland or animal path	85	21
Urban or residential area	177	14
Agricultural field	70	11
Near food processing plant	106	10
Beach or sandbank	24	4
Forest or mountain area	121	2
Footpaths	35	0

L. monocytogenes, all samples from footpaths were negative for *L. monocytogenes*, and only 2% of the samples collected in woodland or mountain areas were positive.

Detection of *L. monocytogenes* correlated with rain and sample humidity. Previous studies have indicated that *L. monocytogenes* is more frequently isolated after recent rainfall, irrigation, and flooding events (7, 8). In the present study, 271 out of 618 samples were collected on days with rainfall and 347 samples on days with no rain within the previous 24 h. When collected on days with rain, 20% of samples were positive for *L. monocytogenes*, while on days with no rain within the last 24 h, only 3% of samples were positive. Thus, our data support previous studies suggesting that prevalence of *L. monocytogenes* is positively associated with rainfall ($P = 2 \times 10^{-12}$ by Fisher's exact test).

Upon sample collection, the humidity of the sampled material was categorized on a scale from 1 (completely dry) to 5 (liquid). Overall, the prevalence of *L. monocytogenes* in samples from the two driest sample categories was 5.6% (4/70) and 5.7% (11/196), while it was 17% (27/164) and 14% (12/86) in the more humid categories 3 and 4. The prevalence was significantly higher in the humid samples (categories 3 and 4) than in the two driest sample categories ($P < 0.02$ by Fisher's exact test). The prevalence in liquid samples (category 5) was 10% (10/102). Among the samples collected in urban environments, the sample humidity was not significantly associated ($P > 0.05$) with the prevalence of *L. monocytogenes*, with an overall prevalence of 10% in categories 1 and 2 (10/92) and 17% in categories 3 to 5 (14/85).

Persistent strains detected in rural and urban environments. To examine whether environmental locations retained their status as *L. monocytogenes* positive or negative over time and whether the same clones were isolated repeatedly from the same location, 70 sites were subjected to one to three additional rounds of sampling the following years. In total, 115 *L. monocytogenes* isolates were collected in the current study (see Table S1 in the supplemental material). All isolates were subjected to WGS, *in silico* MLST, and whole-genome multilocus sequence type (wgMLST) analysis. The distribution of clones (CCs) among the identified isolates is presented in Fig. 2.

Of the 44 sampling points positive for *L. monocytogenes* in the first round of sampling, 28 sites (64%) were positive on at least one of the subsequent sampling occasions. Of the 26 initially negative sites, five turned out positive during later sampling events (19%), and one of these was positive twice. In total, 29 sampling sites were positive for *L. monocytogenes* more than once, and isolates belonging to the same ST were collected repeatedly from seven sites (Table 2). In six cases, STs repeatedly isolated from the same site were very closely related, with a maximum wgMLST allelic distance of 20. When also adjacent or slightly more distant sampling sites (maximum of 3 km) were included, a total of 14 clusters with genetic distances of <20 were repeatedly collected from the same location over periods ranging from 4 months to 3 years (Table S2 and Text S1). When the commonly employed core genome MLST (cgMLST) scheme described by Moura et al. (23) was employed, the isolates could not be distinguished, except in one cluster with distances of 0 to 1 cgMLST alleles. Twelve clusters, including two clusters each for CC91, CC11 (ST451), and CC37, represent clusters of highly similar isolates, with 0 to 8 wgMLST allelic differences. Together, the results strongly indicate

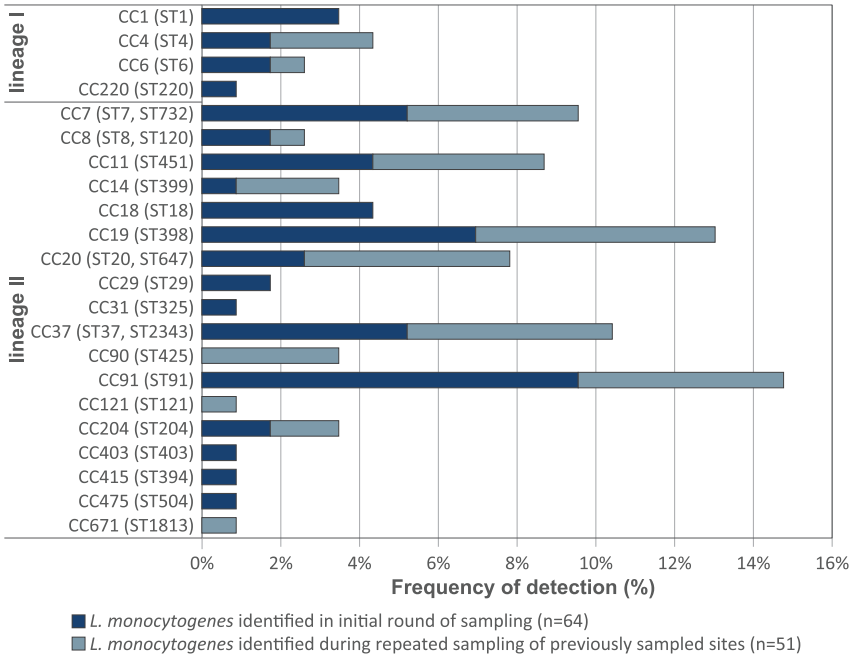

FIG 2 Distribution of CCs among identified isolates from rural and urban environments. The data are reported as percentages of the grand total number of isolates (n = 115). STs represented within each clonal complex (CC) are given in parentheses.

that *L. monocytogenes* clones had persisted in the same environment or were repeatedly reintroduced between sampling events in both rural and urban locations.

We also observed a case where a recent common contamination source was obscure: only 9 wgMLST alleles (and no cgMLST alleles) separated a pair of CC6 isolates found 30 km and 3 years apart; one isolate was from a grazing pasture in Akershus county in 2020, and the other was from soil by the root of a tree in Oslo city center in 2017.

Persistence and cross-contamination on Norwegian dairy farms. In the next step, WGS was performed for a panel of 79 *L. monocytogenes* isolates collected from Norwegian dairy farms (16). A total of 18 dairy herds from four different geographical areas within a 100-km radius from downtown Oslo (Fig. 1b) had each been sampled four to six times between August 2019 and July 2020. Out of the 556 analyzed samples, *L. monocytogenes* was detected in 12 milk filters (13% prevalence), 30 cattle feces samples (30%), 32 samples of cattle feed (silage or silage mixture; 32%), and 5 teat swabs (5%). All bulk tank milk and teat milk samples were negative for *L. monocytogenes*, and for one of the farms (farm 16), all 34 collected samples were negative. An overview of the STs of the collected isolates (Table S1) is presented in Table 3, and a phylogenetic tree showing the genetic relationships between the individual isolates is shown in Fig. 3.

Twelve clusters, each comprising two to four isolates, with pairwise genetic distances in the range of 0 to 11 wgMLST alleles, were isolated from the same farm during repeated multiple visits over periods ranging from 2 to 10 months. These clusters involved 33 of the collected isolates and comprised 10 different CCs (Table S3). These observations strongly support previous studies indicating that the same *L. monocytogenes* clones can persist over time in individual cattle herds or farm environments (15, 35, 36).

Out of 12 isolates from milk filters, four belonged to a persistent cluster and one was closely related to an isolate from a teat swab sample obtained on the same

TABLE 2 STs identified at sampling points positive for *L. monocytogenes* on repeated occasions[a]

Site no.	Sampling point description	2016, Oct	2017, Jun, Oct, Nov	2018, Jun	2018, Sept, Oct	2019, Sept	2020, Jan
Urban or residential area							
33	Brook in residential area	ST451				ST4	
48	Garden compost heap	ST451[a]				ST451[a]	ST425
49	Garden compost heap	ST451				ST425[b]	ST425[b]
66	Puddle next to road	ST20				ST4	
120	Flowerbed in town center		ST91		Negative	ST1813	ST398
121	In front of park bench by flowerbed		ST399		ST451	ST398[c]	ST39[c]
123	Grass lawn in town center		ST398[d]		ST398[d]	Negative	Negative
129	Roadside close to brook		ST37			ST91	Negative
251	Decaying leaves/vegetation on bike path		ST18			ST37[e]	ST37[e]
252	Soil near horse paddock		ST204			ST398	ST7
253	Along sidewalk curb		ST1			Negative	ST425
259	Flowerbed with pigeons, city center		ST204[f]			ST204[f]	ST398
262	At foot of tree, city center		ST6			ST204	ST120
268	Puddle on gravel path, city park		ST6			ST451	Negative
Grazeland or animal path							
53	Decaying vegetation by feeding station	ST4				ST399	ST37
54	Soil close to cattle feeding station	ST91				ST399	ST451
55	Mud close to cattle enclosure	ST37				ST399	ST91
56	Puddle of mud close to cattle enclosure	ST91				ST398	ST6
98	Sheep grazing pasture		ST398		ST451	ST2343	ST91
99	Sheep manure		ST398		Negative	Negative	ST91
100	Animal tracks by feeding station		ST398		Negative	ST37	Negative
101	Animal tracks by feeding station		ST398		Negative	ST91	Negative
130	Soil at edge of pond		ST91		Negative	ST121	Negative
133	Decaying vegetation at edge of pond		ST398		Negative	ST20[g]	Negative
134	Decaying vegetation at edge of pond		ST29		Negative	ST4	ST20[g]
Near food processing plant							
279	Grass next to cold storage entrance		ST732[h]	ST732[h]	ST732[h]		
287	Storm drain outside plant		ST1	Negative	ST647		Negative
363	Gravel from quay outside factory			Negative	ST732		ST647
406	Gravel from quay outside factory				ST1		ST647

[a]Allelic distance between isolates is indicated by the superscript letter: a, 3 wgMLST differences; b, 2 wgMLST differences; c, 2 wgMLST differences; d, 34 wgMLST differences and 0 cgMLST differences; e, 7 wgMLST differences; f, 3 wgMLST differences; g, 0 wgMLST differences (sites 133 and 134 are located 5 m apart); h, 0 to 4 wgMLST differences.

sampling occasion. When the same clone was isolated from several sampling sites at the same farm, the pairwise genetic distances separating milk filter isolates from fecal, feed, or teat swab isolates ranged from 0 to 7 wgMLST allelic differences (Table S3). These links represent likely cross-contamination events where milk filters (and consequently milk) have been contaminated with *L. monocytogenes* clones found in the farm environment.

Detection of closely related isolates from different geographic areas. In four cases, closely related isolates belonging to CC11 (ST451), CC226, and CC415 (ST394) were collected from more than one dairy farm. The genetic differences between isolates from different farms were somewhat greater than the diversity between isolates found on the same farm, with between 9 and 20 pairwise wgMLST allelic differences. The number of cgMLST differences within each cluster was 0 or 1 (Table S3 and Text S1). These data indicate that farms located in different geographical areas host the same strain of *L. monocytogenes*.

Six clusters comprising *L. monocytogenes* from both dairy farms and isolates obtained from rural and urban environments were detected. The genetic distances separating isolates from the two data sets in these clusters ranged from 9 to 27 wgMLST allelic differences and 0 or 1 cgMLST differences (Table S4 and Text S1). The closest link was observed for a cluster of four CC37 isolates; two from grazing land/pasture in the vicinity of Ås and two from feed and teat swab samples obtained on two different visits to farm 12, located about 50 km east of Ås. The two pairs of isolates

TABLE 3 *L. monocytogenes* STs identified on dairy farms[a]

		Farm no.																
Visit date	Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	17	18
Aug/Sept 2019	MF			NS														
	Feces	NS				NS			ST91		ST11	ST4				ST7	ST21	
	Feed					ST226	ST37						ST37				ST6	
	TS															NS		
Nov/Dec 2019	MF	NS		NS	ST451			ST2760	NS			ST451	ST451					
	Feces	ST18			ST16	ST394		ST451			ST204			ST8				
	Feed	ST18			ST226	ST226	ST451	ST37					ST91					
	TS															ST37		
Jan 2020	MF	ST18							NS				ST37					
	Feces	ST18		ST2761									ST451				ST18	
	Feed	ST425	ST394	ST2761		ST7				ST224		ST4	ST451		ST91	ST91	ST18	
	TS			ST20														
Feb/Mar 2020	MF	NS						NS			NS	NS	ST177			NS		
	Feces	ST20		ST2761			ST451				ST21	ST451	ST177		ST91			
	Feed	ST37	ST394				ST451				ST412		ST177		ST91			
	TS	ST20																
May 2020	MF	NS	NS	NS									ST91	ST91			NS	
	Feces	NS	NS	NS									ST91	ST37			NS	
	Feed	NS	NS	NS									ST511	ST8			NS	ST91
	TS	NS	NS	NS									ST37	ST91			NS	
Jun 2020	MF	NS		NS			ST91		ST451	ST451								
	Feces	NS		NS		ST37				ST21	ST412	ST4	ST451	ST124			NS	
	Feed	NS		NS						ST451	ST394		ST177				NS	
	TS	NS	NS	NS					NS								NS	

[a]MF, milk filter; TS, teat swab; NS, not sampled; empty cells, negative for *L. monocytogenes*.

were separated by 9 to 14 wgMLST allelic differences and were indistinguishable by cgMLST.

To further explore the occurrence of genetic links between Norwegian isolates from natural and animal reservoirs, 24 of the 34 *L. monocytogenes* isolates collected from invading slugs (*Arion vulgaris*) from garden and farm environments in Norway by Gismervik et al. (21) were subjected to WGS analysis (Table S1). Interestingly, two pairs of slug isolates collected from different geographic locations showed only 2 (CC14) and 11 (CC1) wgMLST allelic differences. Furthermore, five clusters with 10 to 21 wgMLST allelic differences comprised a slug isolate and one or more isolates from either a rural/urban sampling site or from a dairy farm (Table S5). The closest genetic relationship concerned two CC1 isolates, in which a slug isolate from the west coast of Norway (collected in 2012) showed only 10 wgMLST allelic differences compared to an isolate collected from a street in a residential area in Oslo in 2017.

Thus, counting the previously mentioned pair of CC6 isolates collected in Akershus and Oslo, a total of 17 close genetic links between isolates collected at relatively distant geographic areas in Norway were detected in the set of 218 examined isolates. Presumably, not all clusters represent direct epidemiological links, especially in cases where isolates were collected several years apart. The observed genetic distances within the clusters, $\leq$21 wgMLST and $\leq$3 cgMLST allelic differences, are within the thresholds often suggested as an appropriate guide for defining an outbreak cluster, which is about 7 to 10 cgMLST differences (23, 38, 39) or about 20 single nucleotide polymorphisms (SNPs) in SNP analyses (40, 41), which have a sensitivity comparable to that of wgMLST (34, 42).

Comparison with Norwegian clinical isolates. The identification of close genetic links between isolates from different natural and animal-associated sources without known connections led us to hypothesize that it would be possible to identify clusters containing both environmental and clinical isolates with a similar level of genetic relatedness. A data set of Norwegian clinical isolates was identified, containing 130 genomes from 2010 to 2015 (92% of all reported cases in these years) (43) and two

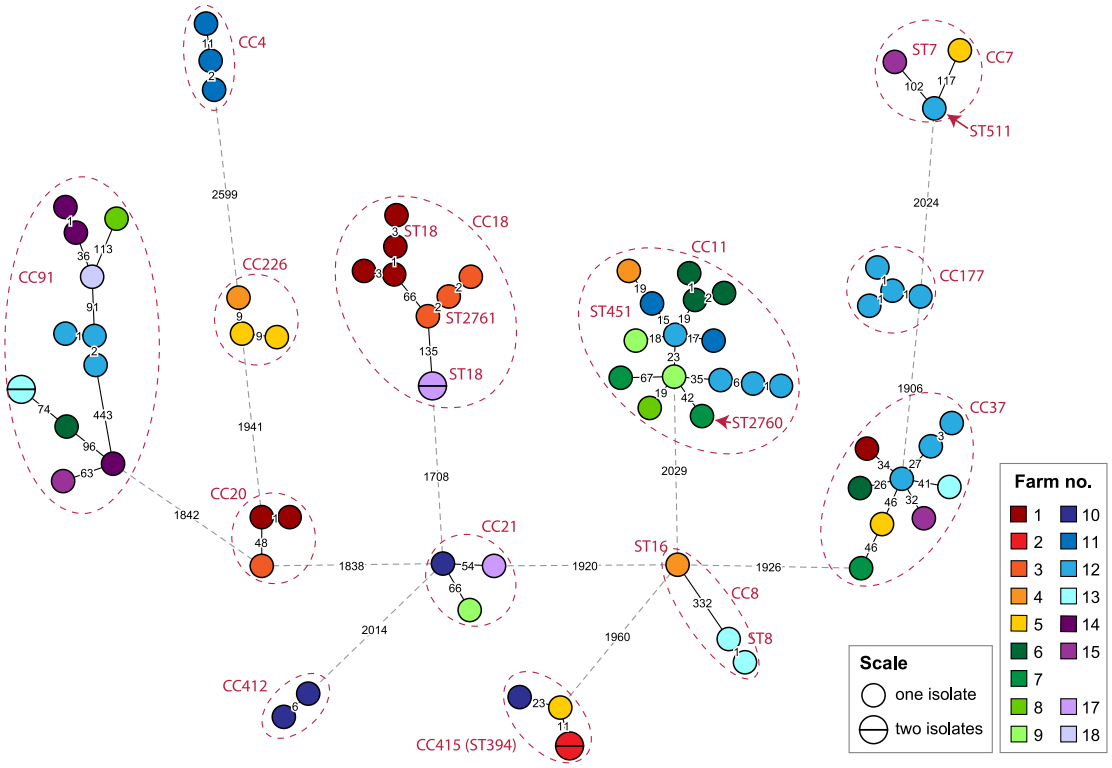

FIG 3 Phylogeny for the *L. monocytogenes* isolates from dairy farms. A minimum spanning tree based on wgMLST analysis is shown. The area of each circle is proportional to the number of isolates represented, and the number of allelic differences between isolates is indicated on the edges connecting two nodes. The CCs and STs are indicated next to each cluster (the CC number is the same as the ST number unless indicated otherwise). Edges shown as dashed lines separate clusters belonging to different clonal complexes. Isolates separated from the nearest other isolate by >1,700 wgMLST alleles (D011L, D058L, D080L, D084L, D144L, and D190L) were excluded from the figure for clarity.

genomes from 2018 (ST20 and ST37), made publicly available by the European Centre for Disease Prevention and Control (ECDC) and the Norwegian Institute of Public Health (NIPH), respectively. Sequencing data of sufficient quality for wgMLST analysis was available for 111 of these isolates (Table S1). An initial comparison between the clinical isolates identified 15 pairs of isolates and nine larger clusters containing 3 to 12 isolates showing genetic distances of ≤10 cgMLST allelic differences (Table S6). Most clusters contained isolates collected during a time span of several years and could represent listeriosis outbreaks or epidemiologically linked cases.

A wgMLST analysis showing the genetic relationships between isolates originating from rural and urban environments, dairy farms, slugs, and clinical cases is shown in Fig. 4 and 5. Nine clusters contained clinical isolates differentiated from isolates sequenced in the current study by genetic distances in the range of 6 to 23 wgMLST allelic differences (0 to 7 cgMLST alleles) (Table S7 and Text S1). The environmental *L. monocytogenes* isolates closely related to clinical isolates were isolates from soil samples from both urban and rural locations (belonging to CC4, CC7, CC11/ST451, CC220, CC403, and CC415/ST394), three slug isolates obtained from garden and farm environments (CC7, CC8, and CC9), and a group of CC11/ST451 isolates from dairy farms. The closest genetic link was found between the single CC9 slug isolate (from 2012) and a clinical isolate from 2015, differentiated by only 6 wgMLST alleles (and 0 cgMLST alleles). The analysis shows that *L. monocytogenes* isolates that are genetically very closely related to clinical isolates can be detected in various natural and agricultural

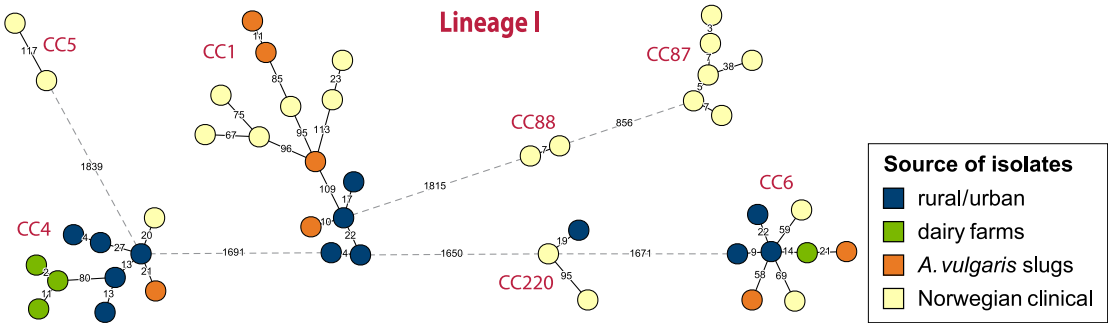

FIG 4 wgMLST phylogeny for *L. monocytogenes* lineage I isolates from Norway. Shown is a minimum spanning tree based on wgMLST analysis. The number of allelic differences between isolates is indicated on the edges connecting two nodes. Edges shown as dashed lines separate clusters belonging to different clonal complexes, which are indicated next to each cluster. Lineage I isolates separated from the nearest other isolate by >900 wgMLST alleles (D084L, ERR2522285, ERR2522267, and ERR2522291) were excluded from the figure for clarity.

environments, even when isolates are collected across timespans ranging several years.

Comparison of prevalence and diversity of MLST clones from different sources. Most isolates from natural and agricultural environments belonged to *L. monocytogenes* lineage II, comprising 89%, 94%, and 68% of isolates from rural/urban environments, dairy farms, and slugs, respectively (Fig. 6a). The remaining isolates belonged to *L. monocytogenes* lineage I, as lineage III or IV isolates were not detected in the current study. The predominant clones among the rural/urban isolates were CC91 (15%), CC19/ST398 (13%), CC37 (10%), and CC11/ST451 (9%). No specific niches were found for these clones, as isolates were spread geographically (3 to 5 counties) and found in 3 to 5 different habitats/areas and in a range of humidity and weather conditions. CC91 appeared most ubiquitous, as it was isolated from five different counties, from different sample types (soil, sand, vegetation, and feces), from five different areas (agricultural fields, urban area, beach, grazeland, and forest), during all seasons, and from all categories of humidity. CC11/ST451, CC91, and CC37 were the most frequently isolated clonal groups at the dairy farms (18%, 15%, and 11%, respectively); each was detected on seven different farms. Among the slug isolates, the most common clones were CC1 (15%) and CC91 (12%) (21). A survey of previous studies indicated that CC1, CC7, and CC37 were the clones most commonly detected in various natural and farm environments (Table S8).

Among the examined Norwegian clinical isolates, CC7 was the most prevalent clonal group, accounting for 23% (n = 30) of the reported listeriosis cases, followed by CC121 (13%), CC8 (8%), and CC1 (6%) (Fig. 6b). In contrast to that observed in many other countries (27, 44), lineage I isolates composed a minority of the clinical isolates in this data set (20). The high prevalence of CC121 among the clinical isolates was unexpected, as this clone is commonly regarded as hypovirulent due to the frequent occurrence of premature stop codons (PMSC) in the gene encoding the virulence factor internalin A (*inlA*) (27), a characteristic also shared by the Norwegian CC121 clinical isolates. Interestingly, the single *L. monocytogenes* CC121 isolated in the current study, MF7617 from soil at the edge of a university campus pond in Ås, had an intact and presumably functional copy of *inlA*. This isolate was only distantly related to the clinical CC121 isolates, separated by 195 wgMLST alleles from the nearest clinical isolate. In contrast to CC121, the other three most commonly detected CCs among the Norwegian clinical isolates, CC1, CC7, and CC8, also were relatively common among the isolates from natural and agricultural environments, with each CC having an average prevalence of between 6% and 7.5% in the rural/urban, dairy farms, and slug isolate data sets (Fig. 6a).

Since listeriosis is primarily acquired from food, the frequency distribution of CCs

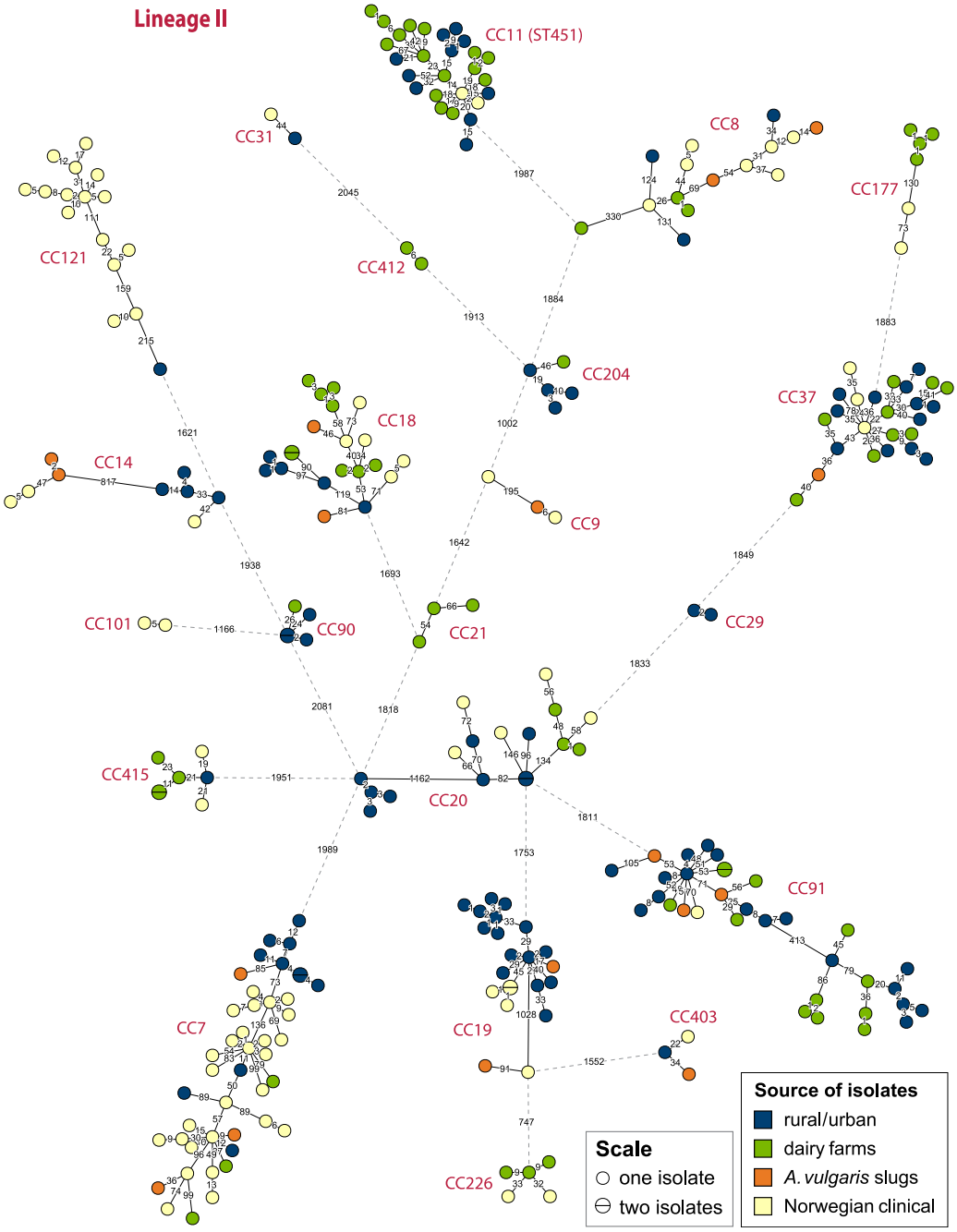

FIG 5 wgMLST phylogeny for *L. monocytogenes* lineage II isolates from Norway. Shown is a minimum spanning tree based on wgMLST analysis. The area of each circle is proportional to the number of isolates represented, and the number of allelic differences between isolates is indicated on the edges connecting two nodes. Edges shown as dashed lines separate clusters belonging to different clonal complexes, which are indicated next to each cluster. Lineage II isolates separated from the nearest other isolate by >900 wgMLST alleles (MF7614, MF6841, D144L, D190L, ERR2522251, and ERR2522298) were excluded from the figure for clarity.

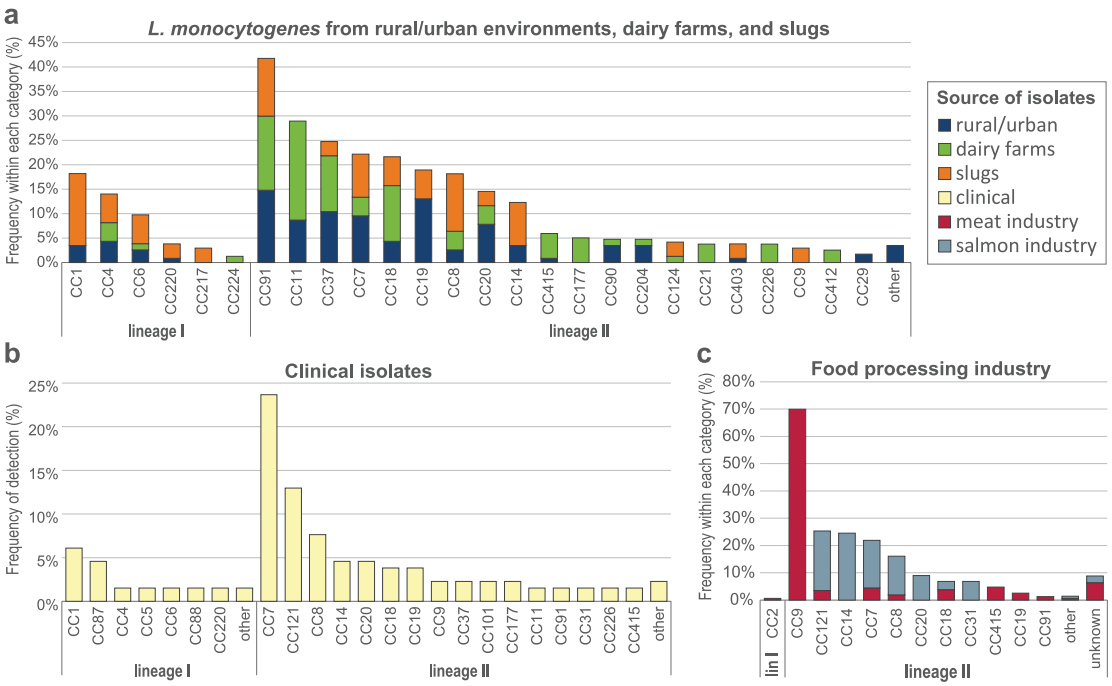

FIG 6 Prevalence and distribution of *L. monocytogenes* MLST clonal complexes (CCs) from different sample types in Norway. The data are reported as percentages of isolates within a given CC in each source category. (a) Prevalence in rural and urban environments (isolated during 2016 to 2020; $n = 115$ isolates), dairy farms (2019 to 2020; $n = 87$), and slugs (2012; $n = 34$). The "other" category comprises one isolate each for CC31, CC121, CC475, and CC671. (b) Prevalence in publicly available genomes from human cases of listeriosis in Norway (2010 to 2015; $n = 129$ and 2018; $n = 2$). The lineage I "other" category comprises a CC3 and a CC59 isolate, and the lineage II "other" category includes one isolate each for CC11, CC101, and CC177. (c) Prevalence within food processing facilities in Norway. The CCs were inferred for isolates from five meat and four salmon processing facilities (meat, 2012 to 2015, $n = 293$; salmon, 2011 to 2014, $n = 358$). The data used to predict the CC for each isolate were multiple-locus variable-number tandem repeat analysis (MLVA) obtained for all isolates and MLST data obtained for representative isolates from each obtained MLVA profile (45). The "unknown" category represents isolates with MLVA profiles identified only once and not subjected to MLST.

for *L. monocytogenes* from the Norwegian food processing industry (Fig. 6c) was estimated from previous work encompassing 680 isolates from five meat and four salmon processing plants, collected during 2011 to 2015 (45). The prevalence of lineage I isolates was <1% among the food processing industry isolates, represented by only two CC2 isolates from the meat industry. In meat processing environments, CC9 was by far the most prevalent clonal group, representing 70% of isolates. It must, however, be noted that most of the collected isolates were from two intensively sampled processing plants (34). One slug isolate and three clinical isolates (2011, 2012, and 2015), but none from dairy farms or samples from rural and urban environments, belonged to CC9. In salmon processing environments, CC14 (ST14) was most prevalent (25%), followed by CC121 (22%), CC7 (ST7, ST732, and ST995; 18%), and CC8 (ST8 and ST551; 14%). CC14/ST14 was only represented by two clinical and two slug isolates and was not detected among isolates from rural/urban environments or dairy farms. The latter three were among the four most prevalent CCs among the Norwegian clinical isolates.

To examine the diversity of the most commonly detected *L. monocytogenes* clonal groups from Norwegian natural environments in an international context, a representative subset of reference genomes belonging to ST37, ST91, and ST451 were selected for comparative analysis using cgMLST (Fig. 7). Of the >6,000 examined publicly available genomes, 243 belonged to one of the three relevant STs. For ST91, a limited number of international reference sequences were available, and nearly 60% of the analyzed isolates were Norwegian. This suggests that this ST is more prevalent in Norway than in

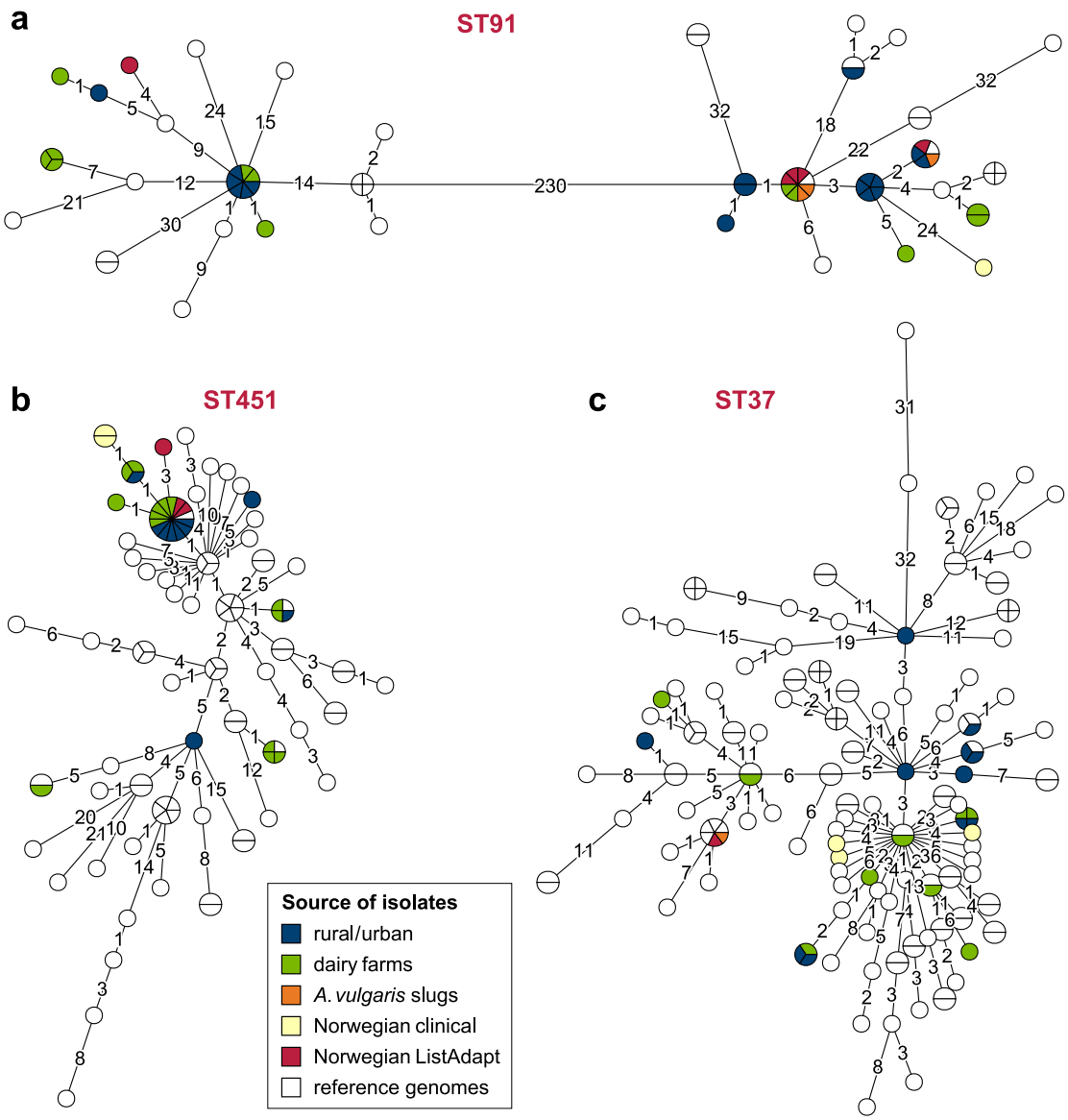

FIG 7 cgMLST phylogeny for the most common STs identified in the current study. Minimum spanning trees based on cgMLST allelic profiles for ST91 (a), ST451 (b), and ST37 (c), showing the relationship between the Norwegian isolates from natural environments, Norwegian clinical isolates, and reference genomes obtained from public databases. Reference genomes were obtained from the BIGSdb-*Lm* database hosted at the Pasteur Institute, WGS data from the EU project ListAdapt (also including genomes from Norwegian sources, labeled in red), and genome assemblies from NCBI GenBank. The area of each circle is proportional to the number of isolates represented, and the number of allelic differences between isolates is indicated on the edges connecting two nodes.

many other countries. For ST37, only limited clustering of Norwegian isolates relative to the international isolates was observed. In contrast, for ST91 and ST451, the Norwegian isolates appeared to cluster with isolates from other countries, indicating that they represent internationally dispersed clones.

DISCUSSION

It has long been acknowledged that *L. monocytogenes* clones predominating among human clinical isolates differ from those that dominate in food (23, 24, 26, 44, 46, 47), and that persistent clones of *L. monocytogenes* may become established in food processing environments (2, 33, 34). Here, we show that the most ubiquitous clones found in soil and other natural and animal ecosystems (CC91, CC11, and CC37) are distinct from clones predominating among both clinical and food isolates, and that *L. monocytogenes* may persist and spread in urban and rural areas, grazeland, agricultural fields, and farm environments. The correspondence of major CCs was high for the three examined sets of environmental isolates (rural/urban, dairy farms, and slugs). CC37 appeared to be exceptionally widespread in natural environments and was isolated from nine different counties and a wide variety of habitats. It was also found to persist for years both at a farm and on a bike path in the capital of Norway. The ubiquity of this clone is also reflected by its detection in a large proportion of other studies investigating the identity of *L. monocytogenes* clones from natural and animal reservoirs, including wildlife, forest areas, and farms (5, 6, 13, 18, 21, 25, 29–31, 48).

The current study identified close genetic relationships between environmental isolates of *L. monocytogenes* collected from geographically and temporally unrelated sources despite a relatively low number of analyzed isolates. Although fixed clustering thresholds for defining outbreak clusters are controversial (37, 49, 50), the genetic distances in the observed clusters were well within the thresholds used to guide outbreak analyses (23, 38–41). In the majority of observed clusters, isolates with no known likely association were indistinguishable using cgMLST analysis, which is the method currently employed for surveillance of *L. monocytogenes* by many laboratories, including the Norwegian Institute of Public Health (51). This finding underscores the need for careful consideration of additional evidence, such as epidemiological data, traceback evidence, and phylogenetic tree topology, as part of WGS-based surveillance and outbreak investigations (52). Ideally, evaluation of possible epidemiological links should consider the occurrence of closely related strains in the whole food chain, including external contamination sources in urban and natural environments (50). Currently, a lack of published genomic data on *L. monocytogenes* from various sources is a barrier for effective management of this pathogen, both for public health authorities and for industrial actors.

During the last decade (2010 to 2020), an average of 24 yearly listeriosis cases have been reported in Norway, and most of them (80%) were domestically acquired (http://www.msis.no/). The implicated food is rarely identified. Only two outbreaks have been publicly reported during this period, both associated with traditional fermented fish (rakfisk), one in 2013 (ST802; four cases in Norway) (53) and one during the winter of 2018 to 2019 (ST20; 12 cases in Norway, 1 in Sweden [54]). A predominance of lineage II was observed among the Norwegian clinical isolates, comprising 80% of isolates during the years 2010 to 2015; an increase relative to the 56% observed during 1992 to 2005 (55). During 2010 to 2015, 71% of listeriosis patients were aged 70 or above, while during 1992 to 2005, only 42% of patients belonged to this high-risk age group (http://www.msis.no/). A distinct feature among Norwegian clinical isolates was the large proportion of CC121 isolates lacking functional internalin A. The only CC121 isolate collected from a natural environment did not have an *inlA* PMSC, supporting the hypothesis that *inlA* mutations constitute an adaptation to food industry environments (56). The relatively high proportion of cases caused by clones of a hypovirulent strain in Norway could be linked to national consumption and storage practices leading to sporadic ingestion of large numbers of the pathogen among high-risk groups.

Worldwide, the hypervirulent clones CC1 and CC4 are significantly more prevalent among clinical isolates than food isolates (27, 57, 58). Together, these two CCs constituted 8% of Norwegian clinical isolates and 11% of the isolates from natural and farm

environments. CC1 and CC4 also appear to be prevalent in natural environments in other countries (13, 29). However, they were not detected in a study of *L. monocytogenes* in nine Norwegian food processing plants (45). Although at least 80% of meat, cheese, and fish consumed in Norway is produced domestically (59), imported processed foods remain a potential source of infections. Notably, however, 45% of Norwegian households report that they hunt, fish or collect bivalve molluscs, and about half of the population grow their own vegetables, herbs, or fruit and collect berries in the wild (60). Furthermore, the current study identified clusters containing closely related isolates from both clinical sources and natural environments despite comparing temporally nonoverlapping sets of isolates. Together, these observations suggest that the relative contribution of industrially processed foods to listeriosis infections is lower in Norway than in other countries.

MATERIALS AND METHODS

Sampling of *L. monocytogenes* from rural and urban environments. Samples were taken to cover what was hypothesized as hot spots and cold spots for *L. monocytogenes* in the outer environment. The sampling plan was designed to cover different geographical regions of Norway and areas hypothesized to have high (urban areas, grazeland, animal paths, and areas near food processing factories) and low (forests and mountain areas, agricultural fields, beaches, and sandbanks) occurrence of *L. monocytogenes*. Samples classified as footpaths were generally from nonurban areas in woods or other areas used for hiking but were separately categorized, as we considered footpaths to be associated with human activities to a greater extent than more pristine woodland or mountain areas. A detailed sampling scheme was prepared, and convenience sampling was performed by people living in or travelling to different areas to cover Norway geographically and to get detailed results from specific areas (e.g., gardens) and local information. The sampling was performed by trained microbiologists informed about the objective of the study and which types of sites should be sampled. When possible, several different suspected hot and cold spots were sampled in the same geographical area, e.g., grazeland and a forest nearby where the cattle did not have access. Sampling was performed year-round except for winter. For a selection of sampling sites, sampling was repeated once or more over a period of 3 years.

The environmental samples (soil, sand, mud, decaying vegetation, surface water, animal dung, etc.) were collected in sterile 50-mL Nunc tubes. All sampling locations were photographed, and GPS coordinates, sample content, habitat/area, and weather conditions were recorded at the time of sample collection. Specific information about the sample was also noted, such as which animals the area was frequently exposed to (e.g., cattle, deer, sheep, and doves) and local information (e.g., popular areas for hiking). The humidity of the collected samples was assessed on a scale from 1 to 5, ranging from completely dry (1) to liquid (5). Samples were stored at 4°C for up to a week before processing, and analyzed according to ISO 11290-1 (61) with selective enrichment in half-Fraser and Fraser broth (Oxoid) and final plating on RAPID'*L.mono* agar (Bio-Rad).

Whole-genome sequencing. For each *L. monocytogenes* isolate from rural/urban environments or from *Arion vulgaris* slugs (21), a single colony was picked, inoculated in 5 mL brain heart infusion broth, and grown at 37°C overnight. Culture samples (1 mL) were lysed using lysing matrix B and a FastPrep instrument (both MP Biomedicals), and genomic DNA was isolated using the DNeasy blood and tissue kit (Qiagen). Libraries for genome sequencing were prepared using the Nextera XT DNA sample preparation kit (Illumina) and sequenced using 2 $\times$ 300 bp reads on a MiSeq instrument (Illumina).

Colonies from the *L. monocytogenes* isolates from dairy farms (16) were inoculated in 20 mL tryptone soy broth and incubated at 37°C for 24 h before 1 mL was pelleted and DNA extracted using the DNeasy blood and tissue kit (Qiagen). Libraries for genome sequencing were prepared using the NEBNext Ultra DNA library prep kit (New England Biolabs) with random fragmentation to 350 bp and sequencing of 2 $\times$ 150 bp on a NovaSeq 6000 S4 flow cell (Illumina).

Genome assembly. All genome assemblies used in phylogenetic analyses were generated as follows. Raw reads were filtered on q15 and trimmed of adaptors before *de novo* genome assembly was performed using SPAdes v3.10.0 or v3.13.0 (62), with the careful option and six *k*-mer sizes (21, 33, 55, 77, 99, and 127). Contigs with sizes of $<$500 bp and with coverage of $<$5 were filtered out. For the *L. monocytogenes* isolates from dairy farms, the genomes released to NCBI GenBank as accession no. PRJNA744724 (see "Data availability," below) were generated using SPAdes v3.14.1 incorporated in the software tool Shovill, available at https://github.com/tseemann/shovill. Shovill also performed adaptor trimming using Trimmomatic, corrected assembly errors, and removed contigs with sizes of $<$500 bp and coverage of $<$2. The quality of all assemblies was evaluated using QUAST v5.0.2 (63) (see results in Table S9 in the supplemental material).

Phylogenetic analyses. Classical MLST analysis followed the MLST scheme described by Ragon et al. (64) and the database maintained at the Institute Pasteur's *L. monocytogenes* online MLST repository (https://bigsdb.pasteur.fr/listeria/). *In silico* MLST typing was performed for raw sequencing data using the program available at https://bitbucket.org/genomicepidemiology/mlst (65) and for genome assemblies using the program available at https://github.com/tseemann/mlst. CCs are defined as groups of ST profiles sharing at least six of seven genes with at least one other member of the group, except for CC14, which is divided into CC14, represented by ST14 and ST399 in the current work, and CC91,

represented by ST91, as isolates belonging to these two groups do not cluster in phylogenetic analyses of *L. monocytogenes* populations (27).

The wgMLST analysis was performed using a whole-genome scheme containing 4,797 coding loci from the *L. monocytogenes* pangenome and the assembly-based BLAST approach, implemented in BioNumerics 7.6 (https://www.bionumerics.com/news/listeria-monocytogenes-whole-genome-sequence-typing). The cgMLST analysis was performed using the scheme described by Moura et al. (23), which is a subscheme of the wgMLST scheme employed in the BioNumerics platform. For publicly available genomes (described below), cgMLST profiles were obtained by sequence query against the BIGSdb-*Lm* cgMLST allele database maintained at the Institut Pasteur (https://bigsdb.pasteur.fr/listeria/). For the genomes sequenced in the current study, cgMLST profiles were extracted from the wgMLST profiles by mapping of the sequences of the cgMLST allele subset to the publicly available nomenclature through synchronization of BioNumerics with the BIGSdb-*Lm* cgMLST allele database. A subset of isolates was subjected to cgMLST analysis using both approaches to confirm that identical cgMLST profiles were obtained. During wgMLST analysis in BioNumerics, each identified unique allele sequence is designated an allele identifier integer. In contrast, for analyses involving the BIGSdb-*Lm* cgMLST allele database, only alleles that are already present in the database will be identified and receive an allele identifier, while novel alleles are recorded as missing loci.

Minimum spanning trees were constructed using BioNumerics based on the categorical differences in the allelic cgMLST or wgMLST profiles for each isolate. The number of allelic differences between isolates was read from genetic distance matrices computed from the absolute number of categorical differences between genomes. Loci with no allele calls were not considered in the pairwise comparison between two genomes. The criterion for inclusion of a cluster in Table S2, Table S3, Table S4, Table S5, and Table S7 was that each genome included in the cluster showed $\leq$20 or $\leq$21 wgMLST allelic differences toward at least one other genome in the cluster. For Table S6, clusters comprising isolates showing $\leq$10 cgMLST allelic differences toward at least one other genome in the cluster were included. Consequently, for clusters with three or more genomes, individual pairs of genomes with genetic distances exceeding the set thresholds were included in the clusters (see also Text S1).

Publicly available genomes. Available genomes of clinical isolates from human patients in Norway were identified by searching the NCBI Pathogen Detection database (https://www.ncbi.nlm.nih.gov/pathogens) on 30 August 2021. Available raw sequencing data from NCBI BioProjects submitted by ECDC and NPIH, accession numbers PRJEB26061 (43) and PRJEB25848, were subjected to *de novo* genome assembly as described for isolates from rural/urban environments. *In silico* MLST genotyping was successful for all genomes except one of the genomes published by the ECDC, and wgMLST analysis was successful for all except 21 of the ECDC genomes.

Reference genomes included in the cgMLST analysis of ST37, ST91, and ST451 genomes were identified from the following selected sources on 27 August 2021: (i) cgMLST profiles from the BIGSdb-*Lm* database (https://bigsdb.pasteur.fr/listeria/), with 15 genomes belonging to relevant STs; (ii) raw WGS data from the ListAdapt project (https://onehealthejp.eu/jrp-listadapt/), containing 1,552 genomes (BioProject no. PRJEB38828); *de novo* genome assembly was performed for the 165 genomes of relevant STs; and (iii) genome assemblies from NCBI GenBank; among the 3,926 *L. monocytogenes* genomes, 63 genomes belonged to the relevant STs.

Data availability. Data from this whole-genome shotgun project have been deposited in the NCBI GenBank database under BioProject numbers PRJNA689486, PRJNA744724, and PRJNA689487. For GenBank and Sequence Read Archive (SRA) accession numbers, see Table S1. The assemblies were annotated using the NCBI Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP) server (http://www.ncbi.nlm.nih.gov/genome/annotation_prok/).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.2 MB.

ACKNOWLEDGMENTS

We thank Merete Rusås Jensen, Anette Wold Åsli, Janina Berg, and Tove Maugesten at Nofima for excellent technical assistance. We also sincerely thank collaborators in the food and salmon processing industry, colleagues at Nofima (Runar Gjerp Solstad, Rasmus Karstad, Halvor Nygaard, and Kristin Skei Nerdal), and Arild Hugo Solstad for contributing to sample collection. We thank Ann-Katrin Llarena (Faculty of Veterinary Medicine, Norwegian University of Life Sciences) for help during genome assembly of *L. monocytogenes* dairy farm isolates. We thank Ida Skaar (Norwegian Veterinary Institute) for providing the *L. monocytogenes* isolates from slugs (21). We thank the team of curators of the Institut Pasteur MLST system (Paris, France) for importing novel alleles, profiles, and/or isolates at http://bigsdb.pasteur.fr/listeria/.

This work was funded by the Norwegian Agriculture and Food Industry Research Funds, grant numbers 262306 and 314743.

REFERENCES

1. Lopez-Valladares G, Danielsson-Tham ML, Tham W. 2018. Implicated food products for listeriosis and changes in serovars of *Listeria monocytogenes* affecting humans in recent decades. Foodborne Pathog Dis 15:387–397. https://doi.org/10.1089/fpd.2017.2419.
2. Ferreira V, Wiedmann M, Teixeira P, Stasiewicz MJ. 2014. *Listeria monocytogenes* persistence in food-associated environments: epidemiology, strain characteristics, and implications for public health. J Food Prot 77:150–170. https://doi.org/10.4315/0362-028X.JFP-13-150.
3. Liao J, Guo X, Weller DL, Pollak S, Buckley DH, Wiedmann M, Cordero OX. 2021. Nationwide genomic atlas of soil-dwelling *Listeria* reveals effects of selection and population ecology on pangenome evolution. Nat Microbiol 6:1021–1030. https://doi.org/10.1038/s41564-021-00935-7.
4. Vivant AL, Garmyn D, Piveteau P. 2013. *Listeria monocytogenes*, a down-to-earth pathogen. Front Cell Infect Microbiol 3:87. https://doi.org/10.3389/fcimb.2013.00087.
5. Linke K, Rückerl I, Brugger K, Karpiskova R, Walland J, Muri-Klinger S, Tichy A, Wagner M, Stessl B. 2014. Reservoirs of *Listeria* species in three environmental ecosystems. Appl Environ Microbiol 80:5583–5592. https://doi.org/10.1128/AEM.01018-14.
6. Terentjeva M, Šteingolde Ž, Meistere I, Elferts D, Avsejenko J, Streikiša M, Gradovska S, Alksne L, Ķibilds J, Bērziņš A. 2021. Prevalence, genetic diversity and factors associated with distribution of *Listeria monocytogenes* and other *Listeria* spp. in cattle farms in Latvia. Pathogens 10:851. https://doi.org/10.3390/pathogens10070851.
7. Weller D, Wiedmann M, Strawn LK. 2015. Spatial and temporal factors associated with an increased prevalence of *Listeria monocytogenes* in spinach fields in New York State. Appl Environ Microbiol 81:6059–6069. https://doi.org/10.1128/AEM.01286-15.
8. Ivanek R, Grohn YT, Wells MT, Lembo AJ, Sauders BD, Wiedmann M. 2009. Modeling of spatially referenced environmental and meteorological factors influencing the probability of *Listeria* species isolation from natural environments. Appl Environ Microbiol 75:5893–5909. https://doi.org/10.1128/AEM.02757-08.
9. Fenlon DR, Wilson J, Donachie W. 1996. The incidence and level of *Listeria monocytogenes* contamination of food sources at primary production and initial processing. J Appl Bacteriol 81:641–650. https://doi.org/10.1111/j.1365-2672.1996.tb03559.x.
10. Lyautey E, Lapen DR, Wilkes G, McCleary K, Pagotto F, Tyler K, Hartmann A, Piveteau P, Rieu A, Robertson WJ, Medeiros DT, Edge TA, Gannon V, Topp E. 2007. Distribution and characteristics of *Listeria monocytogenes* isolates from surface waters of the South Nation River watershed, Ontario, Canada. Appl Environ Microbiol 73:5401–5410. https://doi.org/10.1128/AEM.00354-07.
11. Stea EC, Purdue LM, Jamieson RC, Yost CK, Hansen LT. 2015. Comparison of the prevalences and diversities of *Listeria* species and *Listeria monocytogenes* in an urban and a rural agricultural watershed. Appl Environ Microbiol 81:3812–3822. https://doi.org/10.1128/AEM.00416-15.
12. Weller D, Belias A, Green H, Roof S, Wiedmann M. 2020. Landscape, water quality, and weather factors associated with an increased likelihood of foodborne pathogen contamination of New York streams used to source water for produce production. Front Sustain Food Syst 3:124. https://doi.org/10.3389/fsufs.2019.00124.
13. Raschle S, Stephan R, Stevens MJA, Cernela N, Zurfluh K, Muchaamba F, Nuesch-Inderbinen M. 2021. Environmental dissemination of pathogenic *Listeria monocytogenes* in flowing surface waters in Switzerland. Sci Rep 11:9066. https://doi.org/10.1038/s41598-021-88514-y.
14. Nightingale KK, Schukken YH, Nightingale CR, Fortes ED, Ho AJ, Her Z, Grohn YT, McDonough PL, Wiedmann M. 2004. Ecology and transmission of *Listeria monocytogenes* infecting ruminants and in the farm environment. Appl Environ Microbiol 70:4458–4467. https://doi.org/10.1128/AEM.70.8.4458-4467.2004.
15. Castro H, Jaakkonen A, Hakkinen M, Korkeala H, 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. https://doi.org/10.1128/AEM.02000-17.
16. Idland L, Granquist EG, Aspholm M, Lindbäck T. 2021. 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. agriRxiv https://doi.org/10.31220/agriRxiv.2021.00091.
17. Fox E, O'Mahony T, Clancy M, Dempsey R, O'Brien M, Jordan K. 2009. *Listeria monocytogenes* in the Irish dairy farm environment. J Food Prot 72:1450–1456. https://doi.org/10.4315/0362-028x-72.7.1450.
18. Sauvala M, Woivalin E, Kivisto R, Laukkanen-Ninios R, Laaksonen S, Stephan R, Fredriksson-Ahomaa M. 2021. Hunted game birds–carriers of foodborne pathogens. Food Microbiol 98:103768. https://doi.org/10.1016/j.fm.2021.103768.
19. Wang Y, Lu L, Lan R, Salazar JK, Liu J, Xu J, Ye C. 2017. Isolation and characterization of *Listeria* species from rodents in natural environments in China. Emerg Microbes Infect 6:e44. https://doi.org/10.1038/emi.2017.28.
20. Hellström S, Kiviniemi K, Autio T, Korkeala H. 2008. *Listeria monocytogenes* is common in wild birds in Helsinki region and genotypes are frequently similar with those found along the food chain. J Appl Microbiol 104:883–888. https://doi.org/10.1111/j.1365-2672.2007.03604.x.
21. Gismervik K, Aspholm M, Rørvik LM, Bruheim T, Andersen A, Skaar I. 2015. Invading slugs (*Arion vulgaris*) can be vectors for *Listeria monocytogenes*. J Appl Microbiol 118:809–816. https://doi.org/10.1111/jam.12750.
22. Sauders BD, Overdevest J, Fortes E, Windham K, Schukken Y, Lembo A, Wiedmann M. 2012. Diversity of *Listeria* species in urban and natural environments. Appl Environ Microbiol 78:4420–4433. https://doi.org/10.1128/AEM.00282-12.
23. Moura A, Criscuolo A, Pouseele H, Maury MM, Leclercq A, Tarr C, Björkman JT, Dallman T, Reimer A, Enouf V, Larsonneur E, Carleton H, Bracq-Dieye H, Katz LS, Jones L, Touchon M, Tourdjman M, Walker M, Stroika S, Cantinelli T, Chenal-Francisque V, Kucerova Z, Rocha EPC, Nadon C, Grant K, Nielsen EM, Pot B, Gerner-Smidt P, Lecuit M, Brisse S. 2016. Whole genome-based population biology and epidemiological surveillance of *Listeria monocytogenes*. Nat Microbiol 2:16185. https://doi.org/10.1038/nmicrobiol.2016.185.
24. Painset A, Björkman JT, Kiil K, Guillier L, Mariet JF, Félix B, Amar C, Rotariu O, Roussel S, Perez-Reche F, Brisse S, Moura A, Lecuit M, Forbes K, Strachan N, Grant K, Møller-Nielsen E, Dallman TJ. 2019. LiSEQ–whole-genome sequencing of a cross-sectional survey of *Listeria monocytogenes* in ready-to-eat foods and human clinical cases in Europe. Microb Genom 5:e000257. https://doi.org/10.1099/mgen.0.000257.
25. Félix B, Feurer C, Maillet A, Guillier L, Boscher E, Kerouanton A, Denis M, Roussel S. 2018. Population genetic structure of *Listeria monocytogenes* strains isolated from the pig and pork production chain in France. Front Microbiol 9:684. https://doi.org/10.3389/fmicb.2018.00684.
26. Maury MM, Bracq-Dieye H, Huang L, Vales G, Lavina M, Thouvenot P, Disson O, Leclercq A, Brisse S, Lecuit M. 2019. Hypervirulent *Listeria monocytogenes* clones' adaptation to mammalian gut accounts for their association with dairy products. Nat Commun 10:2488. https://doi.org/10.1038/s41467-019-10380-0.
27. Maury MM, Tsai YH, Charlier C, Touchon M, Chenal-Francisque V, Leclercq A, Criscuolo A, Gaultier C, Roussel S, Brisabois A, Disson O, Rocha EPC, Brisse S, Lecuit M. 2016. Uncovering *Listeria monocytogenes* hypervirulence by harnessing its biodiversity. Nat Genet 48:308–313. https://doi.org/10.1038/ng.3501.
28. Cabal A, Pietzka A, Huhulescu S, Allerberger F, Ruppitsch W, Schmid D. 2019. Isolate-based surveillance of *Listeria monocytogenes* by whole genome sequencing in Austria. Front Microbiol 10:2282. https://doi.org/10.3389/fmicb.2019.02282.
29. Papić B, Pate M, Félix B, Kušar D. 2019. Genetic diversity of *Listeria monocytogenes* strains in ruminant abortion and rhombencephalitis cases in comparison with the natural environment. BMC Microbiol 19:299. https://doi.org/10.1186/s12866-019-1676-3.
30. Fredriksson-Ahomaa M, London L, Skrzypczak T, Kantala T, Laamanen I, Biström M, Maunula L, Gadd T. 2020. Foodborne zoonoses common in hunted wild boars. Ecohealth 17:512–522. https://doi.org/10.1007/s10393-020-01509-5.
31. Sauvala M, Laaksonen S, Laukkanen-Ninios R, Jalava K, Stephan R, Fredriksson-Ahomaa M. 2019. Microbial contamination of moose (*Alces alces*) and white-tailed deer (*Odocoileus virginianus*) carcasses harvested by hunters. Food Microbiol 78:82–88. https://doi.org/10.1016/j.fm.2018.09.011.
32. Castro H, Douillard FP, Korkeala H, Lindström M. 2021. Mobile elements harboring heavy metal and bacitracin resistance genes are common among *Listeria monocytogenes* strains persisting on dairy farms. mSphere 6:e0038321. https://doi.org/10.1128/mSphere.00383-21.

33. Carpentier B, Cerf O. 2011. Review–persistence of *Listeria monocytogenes* in food industry equipment and premises. Int J Food Microbiol 145:1–8. https://doi.org/10.1016/j.ijfoodmicro.2011.01.005.
34. Fagerlund A, Langsrud S, Møretrø T. 2020. In-depth longitudinal study of *Listeria monocytogenes* ST9 isolates from the meat processing industry: resolving diversity and transmission patterns using whole-genome sequencing. Appl Environ Microbiol 86:e00579-20. https://doi.org/10.1128/AEM.00579-20.
35. Haley BJ, Sonnier J, Schukken YH, Karns JS, Van Kessel JA. 2015. Diversity of *Listeria monocytogenes* within a U.S. dairy herd, 2004-2010. Foodborne Pathog Dis 12:844–850. https://doi.org/10.1089/fpd.2014.1886.
36. Latorre AA, Van Kessel JA, Karns JS, Zurakowski MJ, Pradhan AK, Zadoks RN, Boor KJ, Schukken YH. 2009. Molecular ecology of *Listeria monocytogenes*: evidence for a reservoir in milking equipment on a dairy farm. Appl Environ Microbiol 75:1315–1323. https://doi.org/10.1128/AEM.01826-08.
37. Pightling AW, Pettengill JB, Luo Y, Baugher JD, Rand H, Strain E. 2018. Interpreting whole-genome sequence analyses of foodborne bacteria for regulatory applications and outbreak investigations. Front Microbiol 9: 1482. https://doi.org/10.3389/fmicb.2018.01482.
38. Zamudio R, Haigh RD, Ralph JD, De Ste Croix M, Tasara T, Zurfluh K, Kwun MJ, Millard AD, Bentley SD, Croucher NJ, Stephan R, Oggioni MR. 2020. Lineage-specific evolution and gene flow in *Listeria monocytogenes* are independent of bacteriophages. Environ Microbiol 22:5058–5072. https://doi.org/10.1111/1462-2920.15111.
39. Ruppitsch W, Pietzka A, Prior K, Bletz S, Fernandez HL, Allerberger F, Harmsen D, Mellmann A. 2015. Defining and evaluating a core genome multilocus sequence typing scheme for whole-genome sequence-based typing of *Listeria monocytogenes*. J Clin Microbiol 53:2869–2876. https://doi.org/10.1128/JCM.01193-15.
40. Wang Y, Pettengill JB, Pightling A, Timme R, Allard M, Strain E, Rand H. 2018. Genetic diversity of *Salmonella* and *Listeria* isolates from food facilities. J Food Prot 81:2082–2089. https://doi.org/10.4315/0362-028X.JFP-18-093.
41. Allard MW, Strain E, Rand H, Melka D, Correll WA, Hintz L, Stevens E, Timme R, Lomonaco S, Chen Y, Musser SM, Brown EW. 2019. Whole genome sequencing uses for foodborne contamination and compliance: discovery of an emerging contamination event in an ice cream facility using whole genome sequencing. Infect Genet Evol 73:214–220. https://doi.org/10.1016/j.meegid.2019.04.026.
42. Jagadeesan B, Baert L, Wiedmann M, Orsi RH. 2019. Comparative analysis of tools and approaches for source tracking *Listeria monocytogenes* in a food facility using whole-genome sequence data. Front Microbiol 10:947. https://doi.org/10.3389/fmicb.2019.00947.
43. Van Walle I, Bjorkman JT, Cormican M, Dallman T, Mossong J, Moura A, Pietzka A, Ruppitsch W, Takkinen J, European Listeria Wgs Typing Group. 2018. Retrospective validation of whole genome sequencing-enhanced surveillance of listeriosis in Europe, 2010 to 2015. Euro Surveill 23: 1700798. https://doi.org/10.2807/1560-7917.ES.2018.23.33.1700798.
44. Chenal-Francisque V, Lopez J, Cantinelli T, Caro V, Tran C, Leclercq A, Lecuit M, Brisse S. 2011. Worldwide distribution of major clones of *Listeria monocytogenes*. Emerg Infect Dis 17:1110–1112. https://doi.org/10.3201/eid/1706.101778.
45. Møretrø T, Schirmer BCT, Heir E, Fagerlund A, Hjemli P, Langsrud S. 2017. Tolerance to quaternary ammonium compound disinfectants may enhance growth of *Listeria monocytogenes* in the food industry. Int J Food Microbiol 241:215–224. https://doi.org/10.1016/j.ijfoodmicro.2016.10.025.
46. Henri C, Leekitcharoenphon P, Carleton HA, Radomski N, Kaas RS, Mariet JF, Felten A, Aarestrup FM, Gerner Smidt P, Roussel S, Guillier L, Mistou MY, Hendriksen RS. 2017. An assessment of different genomic approaches for inferring phylogeny of *Listeria monocytogenes*. Front Microbiol 8:2351. https://doi.org/10.3389/fmicb.2017.02351.
47. Gray MJ, Zadoks RN, Fortes ED, Dogan B, Cai S, Chen Y, Scott VN, Gombas DE, Boor KJ, Wiedmann M. 2004. *Listeria monocytogenes* isolates from foods and humans form distinct but overlapping populations. Appl Environ Microbiol 70:5833–5841. https://doi.org/10.1128/AEM.70.10.5833-5841.2004.
48. Kim SW, Haendiges J, Keller EN, Myers R, Kim A, Lombard JE, Karns JS, Van Kessel JAS, Haley BJ. 2018. Genetic diversity and virulence profiles of *Listeria monocytogenes* recovered from bulk tank milk, milk filters, and milking equipment from dairies in the United States (2002 to 2014). PLoS One 13: e0197053. https://doi.org/10.1371/journal.pone.0197053.
49. Schürch AC, Arredondo-Alonso S, Willems RJL, Goering RV. 2018. Whole genome sequencing options for bacterial strain typing and epidemiologic analysis based on single nucleotide polymorphism versus gene-by-gene-based approaches. Clin Microbiol Infect 24:350–354. https://doi.org/10.1016/j.cmi.2017.12.016.
50. Gerner-Smidt P, Besser J, Concepción-Acevedo J, Folster JP, Huffman J, Joseph LA, Kucerova Z, Nichols MC, Schwensohn CA, Tolar B. 2019. Whole genome sequencing: bridging one-health surveillance of foodborne diseases. Front Public Health 7:172. https://doi.org/10.3389/fpubh.2019.00172.
51. Løvdal T, Brandal LT, Sundaram AYM, Naseer U, Roth B, Lunestad BT. 2021. Small-scale comparative genomic analysis of *Listeria monocytogenes* isolated from environments of salmon processing plants and human cases in Norway. Hygiene 1:43–55. https://doi.org/10.3390/hygiene1010005.
52. Brown E, Dessai U, McGarry S, Gerner-Smidt P. 2019. Use of whole-genome sequencing for food safety and public health in the United States. Foodborne Pathog Dis 16:441–450. https://doi.org/10.1089/fpd.2019.2662.
53. Nielsen EM, Björkman JT, Kiil K, Grant K, Dallman T, Painset A, Amar C, Roussel S, Guillier L, Félix B, Rotariu O, Perez-Reche F, Forbes K, Strachan N. 2017. Closing gaps for performing a risk assessment on Listeria monocytogenes in ready-to-eat (RTE) foods: activity 3, the comparison of isolates from different compartments along the food chain, and from humans using whole genome sequencing (WGS) analysis. EFSA Support Pub 14:1151E. https://doi.org/10.2903/sp.efsa.2017.EN-1151.
54. Public Health Agency of Sweden (Folkhälsomyndigheten). 2020. Listerios 2019. Sammanfattning och bedömning. https://www.folkhalsomyndigheten.se/folkhalsorapportering-statistik/statistik-a-o/sjukdomsstatistik/listeriainfektion/kommentarer-och-specialstatistik/2019/. Accessed 7 September 2021.
55. Rosef O, Kræbøe H, Paulauskas A, Ambrasiene D. 2012. Diversity of *Listeria monocytogenes* isolated from humans, food, and environmental sources in Norway. Vet Med Zoot 59:71–79.
56. Harrand AS, Jagadeesan B, Baert L, Wiedmann M, Orsi RH. 2020. Evolution of *Listeria monocytogenes* in a food processing plant involves limited single-nucleotide substitutions but considerable diversification by gain and loss of prophages. Appl Environ Microbiol 86:e02493-19. https://doi.org/10.1128/AEM.02493-19.
57. Lee S, Chen Y, Gorski L, Ward TJ, Osborne J, Kathariou S. 2018. *Listeria monocytogenes* source distribution analysis indicates regional heterogeneity and ecological niche preference among serotype 4b clones. mBio 9: e00396-18. https://doi.org/10.1128/mBio.00396-18.
58. Kwong JC, Mercoulia K, Tomita T, Easton M, Li HY, Bulach DM, Stinear TP, Seemann T, Howden BP. 2016. Prospective whole-genome sequencing enhances national surveillance of *Listeria monocytogenes*. J Clin Microbiol 54:333–342. https://doi.org/10.1128/JCM.02344-15.
59. Helsedirektoratet. 2021. Utviklingen i norsk kosthold 2020. Matforsyningsstatistikk. https://www.helsedirektoratet.no/rapporter/utviklingen-i-norsk-kosthold. Accessed 21 October 2021.
60. Bugge AB. 2015. Moralities of food and meals–how to eat properly. Statens Institutt for Forbruksforskning (SIFO), SIFO-report no. 3–2015. https://hdl.handle.net/20.500.12199/927.
61. International Organization of Standardization. 2017. Microbiology of the food chain–horizontal method for the detection and enumeration of *Listeria monocytogenes* and of Listeria spp.—Part 1. Detection method (ISO 11290–1). https://www.iso.org/standard/60313.html.
62. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Son P, Prjibelski AD, Pyshkin AV, Sirotkin AV, Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA. 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 19:455–477. https://doi.org/10.1089/cmb.2012.0021.
63. Mikheenko A, Prjibelski A, Saveliev V, Antipov D, Gurevich A. 2018. Versatile genome assembly evaluation with QUAST-LG. Bioinformatics 34: i142–i150. https://doi.org/10.1093/bioinformatics/bty266.
64. Ragon M, Wirth T, Hollandt F, Lavenir R, Lecuit M, Le Monnier A, Brisse S. 2008. A new perspective on *Listeria monocytogenes* evolution. PLoS Pathog 4:e1000146. https://doi.org/10.1371/journal.ppat.1000146.
65. Larsen MV, Cosentino S, Rasmussen S, Friis C, Hasman H, Marvig RL, Jelsbak L, Sicheritz-Ponten T, Ussery DW, Aarestrup FM, Lund O. 2012. Multilocus sequence typing of total-genome-sequenced bacteria. J Clin Microbiol 50:1355–1361. https://doi.org/10.1128/JCM.06094-11.
66. Kahle D, Wickham H. 2013. ggmap: spatial visualization with ggplot2. R J 5:144–161. https://doi.org/10.32614/RJ-2013-014.

Supplemental Material
S1 Text

Additional information regarding clusters of closely related *L. monocytogenes* isolates.

WGS analysis of *Listeria monocytogenes* from rural, urban, and farm environments in Norway: Genetic diversity, persistence, and relation to clinical and food isolates

Annette Fagerlund[a]#, Lene Idland[b], Even Heir[a], Trond Møretrø[a], Marina Aspholm[b], Toril Lindbäck[b], and Solveig Langsrud[a]

[a] Nofima, Norwegian Institute of Food, Fisheries and Aquaculture Research, Ås, Norway

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

Table of Contents

Persistent strains detected in rural and urban environments

Persistence events in rural and urban environments were revealed by WGS analysis (S2 Table). Sampling in a private garden compost heap on three occasions resulted in seven positive samples of *L. monocytogenes*. Four isolates identified in 2016 and 2019 belonged to ST451 (CC11), and three ST425 (CC90) isolates were from 2019 and 2020. The ST451 isolates differed by 1 to 10 wgMLST allelic differences, with 2 to 9 alleles distinguishing the 2019 isolate from the three isolates from 2016. In the ST425 cluster, two isolates from 2020 were indistinguishable by wgMLST but differed by 2 alleles relative to the strain isolated 4 months earlier, in 2019.

Sampling in the town centre of Ås and the nearby NMBU university campus (Akershus) in September 2019 and January 2020 also resulted in detection of several clones that were repeatedly collected from the same sampling site. One was a ST398 (CC19) clone, first isolated in front of a park bench by a flower bed in 2019. In 2020, an isolate from the same location and an isolate from the adjacent flower bed were found, with all three isolates differing from each other by 2 wgMLST alleles. Two ST20 (CC20) isolates, indistinguishable by wgMLST, were collected in 2019 and 2020, from two sampling sites by the university campus pond, located only 5 meters apart. Two isolates belonging to ST204 (CC204), separated by only 3 allelic differences, were collected two years apart (2017 and 2019) from the same flowerbed next to the entrance to a subway station in central Oslo. Sampling in Oslo also resulted in detection of two ST37 (CC37) isolates differing by 7 alleles, isolated 4 months apart from samples of sand/gravel on a bike path next to a major road.

In some cases, isolates belonging to the same clone were collected from nearby locations, but not at the exact same sampling site. For example, sampling in rural areas in the vicinity of Ås resulted in detection of a cluster of two pairs of ST91 (CC91) isolates collected 4 months apart, which were separated by 2 to 5 wgMLST allelic differences. One of the isolates was found ~500 meters north of the area where the remaining three were found, but both sites were used as pasture for sheep. It is, therefore, possible that the same herd of sheep may have grazed and shed *L. monocytogenes* at both locations. Interestingly, two years earlier, a related isolate differentiated from this cluster by 11 to 12 allelic differences was collected from a flower bed in Ås town centre, around 2 km to the southwest. Furthermore, two isolates, belonging to a different clade within ST91 and separated by only 8 allelic differences, were collected in 2016 and 2017 at sampling sites separated by 1.5 km in the Ås area.

Sampling in a port town in western Norway, in an area with operational fish processing industry, resulted in isolation of several clones of *L. monocytogenes* which were identified during multiple occasions. During a period of almost 1 year, two isolates belonging to ST1 (CC1) differing by only 4 wgMLST alleles, and eight isolates belonging to ST732 (CC7), differing by 0 to 23 wgMLST alleles, were collected at in different locations on the quay. The source of these isolates is likely to be the fishing industry, since the same clones were also identified within the fish processing plant located in the same area (data not shown). However, four isolates belonging to ST647 (CC20), which had not been detected inside the factory, were also isolated from the same area. These four isolates, two from 2018 and two from 2020, differed by 2 to 3 alleles.

Persistence and cross-contamination on Norwegian dairy farms

Potential cross-contamination events on the dairy farms, e.g., contamination of milk (assessed by sampling milk filters) from feed or feces found on the same farm, were revealed by WGS analysis (S3 Table). Notably, only three out of 12 isolates from milk filters were closely related (1-3 wgMLST allelic differences) to fecal and/or feed isolates obtained during the same sampling occasion at the same farm (farms 1 and 12). In all three cases, the same clone (max 5 wgMLST allelic differences) was isolated from feed samples also during subsequent sampling occasions. Furthermore, the same ST91

clone – identical by wgMLST analysis – was collected from a milk filter and a teat swab during the same sampling occasion (farm 13). A ST451 clone found on a milk filter on the second visit to farm 12 was found to be closely related (6-7 allelic differences) to isolates from feed and feces samples collected during a later farm visit. We also observed a case where two ST451 isolates from a milk filter and from feed collected during the same visit to farm 9 were less likely to be from the same contamination source, as the two isolates differed by 28 wgMLST alleles. Nevertheless, it seems clear that milk filters (and consequently milk) may become contaminated with *L. monocytogenes* clones found in the farm environment.

Out of the 19 visits to a farm where both the feed and feces samples were positive for *L. monocytogenes*, the same clone (0-3 wgMLST allelic differences) was found in both samples on seven occasions. An additional four cases were identified where the same clone (0-3 allelic differences) was found on a milk filter or teat swab and in feed or feces samples, or in both milk filter and teat swab samples, on the same visit. Six of these clones were also isolated during more than one visit to the same farm. All in all, we found 12 pairs or clusters of isolates that were repeatedly isolated from the same farm, with pairwise allelic differences within each cluster ranging from 0 to 11 wgMLST alleles. The interval between visits ranged from 2 to 10 months. Four of the clusters were found on Farm 12, which was the farm with the highest detection rate for *L. monocytogenes* with 15 positive samples in total. The remaining eight repeatedly isolated clones were collected on eight different farms. Together, these findings strongly suggest that the same *L. monocytogenes* clones can persist over time in dairy farm environments. The identified persistent clones belonged to ST4, ST8, ST18 and ST2761 (both CC18), ST37, ST91, ST177, ST226, ST394 (CC415), ST412, and ST451 (CC11; two clusters).

Detection of closely related isolates from different geographic areas

Four cases where the same clone was collected on more than one farm were identified (S3 Table). In the first case, two identical ST394 (CC415) isolates collected from feed samples from Farm 2 in January and February 2020 showed 11 wgMLST allelic differences towards an isolate collected from feces at Farm 5 in November 2019. The feed sample taken on the same visit at Farm 5 was positive for ST226, not ST394. In the second case, this ST226 isolate from feed from Farm 5 showed 9 wgMLST allelic differences towards a feed sample from the same farm isolated three months earlier, and 12 allelic differences towards an isolate from feed at Farm 4 also obtained in November 2019. The three farms were located in the same geographical area of Oppland county. In the third case, involving ST451 (CC11) and Farms 8 and 9, located in Akershus county, a milk filter isolate from one farm showed 19 allelic differences towards a feed sample from the second farm. The fourth case also involved ST451 and a total of eight strains; a cluster of three isolates from feed and feces from Farm 6, and five isolates obtained from milk filter and feces samples on four other farms. The number of allelic differences between these isolates (not considering the differences within the Farm 6 cluster) ranged from 15 to 57, with isolates from all farms linked by 20 or fewer pairwise allelic differences. The farms were not located in the same geographical area (Østfold, Akershus and Oppland). These data suggest that farms located at different geographical areas may host the same genetic clones of *L. monocytogenes*. Although the diversity between clones found on different farms was somewhat greater than the diversity between clones found on the same farm, the isolates from different farms could in most cases not be distinguished using the commonly employed core genome MLST (cgMLST) analysis.

A total of six clusters were identified containing *L. monocytogenes* isolates from both rural or urban environments and dairy farms, with genetic distances ranging from 9 to 27 wgMLST allelic differences, and 0 or 1 cgMLST differences (S4 Table). Several of the links involved isolates obtained

from livestock grazeland in the Ås area. The closest link was observed between two ST37 isolates collected in the vicinity of Ås. These samples were collected 3 years and 1.5 km apart and differed by only 3 alleles. Both locations were grazing land/pasture, with one of the locations also used as a feeding location for livestock. These two isolates were closely related to two isolates from feed and teat swab samples obtained on two different visits to farm 12, about 50 km from Ås, with 9-14 wgMLST allelic differences between the pairs of isolates. In another ST37 cluster, an isolate from feed from dairy farm 1, located north of Oslo, showed 15 and 16 wgMLST allelic differences towards two linked isolates from grazing land/pasture from an area in southwestern Norway. Similarly, the previously described ST91 cluster, consisting of four isolates collected in the period 2019-2020 from grazing land close to Ås, showed 20 or 21 allelic differences compared to an isolate from feed at farm 18, located about 100 km north-west of Ås. Furthermore, an ST6 isolate collected from a feed sample from dairy farm 17, located about 70 km west of Ås, differed by 14 wgMLST alleles from an isolate collected from grazing land at Ås in 2020. These two isolates were part of a cluster also containing an isolate collected next to a tree in central Oslo in 2017, and which differed from the two other isolates with 18 and 9 alleles, respectively. Two additional cases where dairy farm isolates differed from strains from the rural/urban area dataset with about 20 allelic differences were also found. The first was the cluster of three ST394 isolates from Farms 2 and 5, which showed 21 or 23 wgMLST allelic differences towards an isolate from farmland in northern Norway from 2018. The second involved the cluster of eight ST451 strains from five different farms, which differed by between 15 and 27 wgMLST alleles from the cluster of ST451 isolates from the garden compost heap in Ås.

Genetic distances between clinical and environmental isolates

Nine clusters containing both one or more clinical isolates and one or more isolates originating from rural and urban environments, dairy farms, or slugs is presented in S7 Table. One cluster belonged to CC8 (ST8) and two belonged to CC7 (ST7). The ST8 cluster comprised a slug isolate from 2012 with 14-15 allelic differences towards two clinical isolates from 2012 and 2013. Within ST7, the first cluster contained three clinical isolates from 2010 and 2015 differing by 10 to 17 wgMLST alleles. This cluster also contained an isolate from slugs with 9 to 18 allelic differences towards the three clinical isolates, and an isolate taken in the vicinity of a horse paddock in Oslo in 2020, showing 12 to 21 differences towards the clinical isolate trio. The other ST7 cluster was composed of five closely related clinical isolates, one from 2010 and the remaining four from 2012, separated by only 1 to 6 allelic differences. This cluster was genetically associated, through allelic differences ranging from 11 to 15, with a single isolate from 2020 obtained from a sample taken in the woods in the vicinity of a meatpacking factory. Strikingly, there was another relatively good match between an isolate taken in the vicinity of this factory and a clinical isolate. These were ST220 isolates linked by 19 wgMLST allelic differences, one obtained in 2020 from the road leading to the factory and the other a clinical isolate from 2013. The two Norwegian clinical CC11 (ST451) isolates – both from 2013 and separated by 2 allelic differences – were linked by 9 and 11 wgMLST allelic differences to an isolate from 2019 obtained from a milk filter on a dairy farm. These three isolates were part of a larger cluster of relatively closely related ST451 isolates – with seven additional dairy farm isolates and six isolates from rural or urban locations separated from the two clinical isolates by distances ranging from 14 to 23 wgMLST alleles.

Regarding differences in genetic distances between cgMLST and wgMLST analyses

The cgMLST scheme (1748 loci) is a subscheme of the wgMLST scheme (4797 loci in total). In S6 Table, clusters of clinical isolates showing of ≤10 cgMLST allelic differences towards at least one other genome in the cluster were included. When the genomes in each of these clusters were analysed using wgMLST, the genetic distances within these clusters ranged from 2 to 105. In S2-S4

Tables and S7 Table, clusters of genomes showing maximum 20-21 wgMLST allelic differences towards at least one other genome in the cluster were included. Also in these clusters, there were several examples of clusters with few cgMLST allelic differences and a relatively large range of variable genes in the wgMLST analysis.

Two factors contributed to the large difference in variable genes obtained using cgMLST analysis relative to wgMLST analysis observed in a subset of the clusters. Both factors were associated with the fact that during pairwise comparison between two genomes, a locus is not called as variable if one of the genomes does not have an allele call for this locus.

Firstly, several cgMLST loci in which the alleles differed between genomes were not recorded as such because the allele found in one or more of the genomes in a cluster was not present in the BIGSdb-*Lm* cgMLST allele database. This effect could be alleviated by a greater representation of genomes in the BIGSdb-*Lm* cgMLST isolate database.

Secondly, the cgMLST scheme containing core loci per definition does not contain variable genetic elements, while the wgMLST scheme contains stable loci from the accessory genome, including loci in prophage regions. If one genome in a cluster lacks a certain prophage, these loci will (correctly) not be called in the wgMLST analysis. However, if two or more other genomes do show allelic variations in these prophage genes, the range of pairwise wgMLST distances between genomes in the cluster can become relatively large. Each genome containing the prophage is nevertheless linked to the cluster by a small number of genetic differences towards the genome(s) lacking the prophage(s) in question.

Examples:

The two CC177 genomes in **S6 Table Cluster 13** (ERR2522308, ERR2522327) differed by 73 wgMLST alleles but only 2 cgMLST alleles. For 32 of the differences called by wgMLST, the locus was part of the cgMLST subscheme but only called in one genome in the pair in the cgMLST analysis. Of the 39 differing wgMLST loci that were not part of the cgMLST subscheme, 17 were located in regions identified as prophage sequences using the PHASTER phage search tool (https://phaster.ca/).

Cluster 24 in S3 Table contains eight genomes belonging to CC11/ST451. The genetic distance within the cluster (not considering the differences between the three closely related isolates D118L, D117L, D044L from Farm 6) ranged from 0-1 cgMLST alleles and 15-57 wgMLST alleles. In total, 105 wgMLST loci showed variable alleles in at least one pair of genomes, and of these, 36 loci belonged to the cgMLST subscheme. Overall, 34 of the differing cgMLST loci were not reported as variable in the cgMLST analysis because the least frequent allelic variant was not present in the BIGSdb-*Lm* cgMLST allele database. Of the 69 variable loci not present in the cgMLST subscheme, 44 were located in regions identified as prophage sequences using the PHASTER phage search tool, and one locus belonged to a plasmid.

The eight CC7 genomes in **S6 Table Cluster 23** differed by 0-10 cgMLST alleles and by 1-105 wgMLST alleles. A total of 176 wgMLST loci were called as variable among the genomes in the cluster, of which 86 belonged to the cgMLST subscheme. Of these, 76 were not reported as variable in the cgMLST analysis because the least frequent allelic variant was not present in the BIGSdb-*Lm* cgMLST allele database. Of the 90 variable loci not present in the cgMLST subscheme, 39 were located in regions identified as prophage sequences using the PHASTER phage search tool, and one locus belonged to a plasmid.

Supplemental S1 Table: Isolates included in the current study

Isolate	Category	wgMLST performed	MLST	CC	Lineage	NCBI BioProject	BioSample Accession no.	GenBank Accession no.	SRA Run Accession no.	Sampling point (rural/urban envs) or dairy farm number	Habitat or sampling area, or sample type	Sampling date	Geographic area
D001L	dairy farm	yes	ST-226	CC226	II	PRJNA744724	SAMN20115069	JAHVVK000000000	SRR17225292	Farm 5	feed	August 2019	Oppland
D006L	dairy farm	yes	ST-37	CC37	II	PRJNA744724	SAMN20115070	JAHVVL000000000	SRR17225291	Farm 6	feed	August 2019	Akershus
D007L	dairy farm	yes	ST-91	CC91	II	PRJNA744724	SAMN20115071	JAHVVM000000000	SRR17225280	Farm 8	feces	August 2019	Akershus
D010L	dairy farm	yes	ST-4	CC4	I	PRJNA744724	SAMN20115072	JAHVVN000000000	SRR17225269	Farm 11	feces	September 2019	Østfold
D011L	dairy farm	yes	ST-6	CC6	I	PRJNA744724	SAMN20115073	JAHVVO000000000	SRR17225258	Farm 17	feed	September 2019	Buskerud
D018L	dairy farm	yes	ST-7	CC7	II	PRJNA744724	SAMN20115075	JAHVVQ000000000	SRR17225236	Farm 15	feces	September 2019	Buskerud
D029L	dairy farm	yes	ST-37	CC37	II	PRJNA744724	SAMN20115076	JAHVVR000000000	SRR17225225	Farm 12	feed	September 2019	Østfold
D031La	dairy farm	yes	ST-21	CC21	II	PRJNA744724	SAMN20115077	JAHVVS000000000	SRR17225214	Farm 17	feces	September 2019	Buskerud
D031Lb	dairy farm	yes	ST-18	CC18	II	PRJNA744724	SAMN20115078	JAHVVT000000000	SRR17225211	Farm 1	feces	November 2019	Oppland
D032L	dairy farm	yes	ST-18	CC18	II	PRJNA744724	SAMN20115079	JAHVVU000000000	SRR17225290	Farm 1	feed	November 2019	Oppland
D034L	dairy farm	yes	ST-451	CC11	II	PRJNA744724	SAMN20115081	JAHVVW000000000	SRR17225288	Farm 4	milk filter	November 2019	Oppland
D035L	dairy farm	yes	ST-16	CC8	II	PRJNA744724	SAMN20115082	JAHVVX000000000	SRR17225287	Farm 4	feces	November 2019	Oppland
D036L	dairy farm	yes	ST-226	CC226	II	PRJNA744724	SAMN20115083	JAHVVY000000000	SRR17225286	Farm 4	feed	November 2019	Oppland
D037L	dairy farm	yes	ST-394	CC415	II	PRJNA744724	SAMN20115084	JAHVVZ000000000	SRR17225285	Farm 5	feces	November 2019	Oppland
D038L	dairy farm	yes	ST-226	CC226	II	PRJNA744724	SAMN20115085	JAHVWA000000000	SRR17225284	Farm 5	feed	November 2019	Oppland
D044L	dairy farm	yes	ST-451	CC11	II	PRJNA744724	SAMN20115086	JAHVWB000000000	SRR17225283	Farm 6	feed	November 2019	Akershus
D045L	dairy farm	yes	ST-2760	CC11	II	PRJNA744724	SAMN20115087	JAHVWC000000000	SRR17225282	Farm 7	milk filter	November 2019	Akershus
D046L	dairy farm	yes	ST-37	CC37	II	PRJNA744724	SAMN20115088	JAHVWD000000000	SRR17225281	Farm 7	feed	November 2019	Akershus
D049L	dairy farm	yes	ST-451	CC11	II	PRJNA744724	SAMN20115089	JAHVWE000000000	SRR17225279	Farm 7	feces	November 2019	Akershus
D050L	dairy farm	yes	ST-451	CC11	II	PRJNA744724	SAMN20115090	JAHVWF000000000	SRR17225278	Farm 11	milk filter	November 2019	Østfold
D051L	dairy farm	yes	ST-91	CC91	II	PRJNA744724	SAMN20115091	JAHVWG000000000	SRR17225277	Farm 12	feed	November 2019	Østfold
D052L	dairy farm	yes	ST-8	CC8	II	PRJNA744724	SAMN20115092	JAHVWH000000000	SRR17225276	Farm 13	feces	November 2019	Østfold
D058L	dairy farm	yes	ST-204	CC204	II	PRJNA744724	SAMN20115093	JAHVWI000000000	SRR17225275	Farm 10	feces	November 2019	Østfold
D066L	dairy farm	yes	ST-451	CC11	II	PRJNA744724	SAMN20115094	JAHVWJ000000000	SRR17225274	Farm 12	milk filter	November 2019	Østfold
D067L	dairy farm	yes	ST-37	CC37	II	PRJNA744724	SAMN20115095	JAHVWK000000000	SRR17225273	Farm 15	teat swab	December 2019	Buskerud
D075L	dairy farm	yes	ST-20	CC20	II	PRJNA744724	SAMN20115096	JAHVWL000000000	SRR17225272	Farm 3	teat swab	January 2020	Oppland
D076L	dairy farm	yes	ST-7	CC7	II	PRJNA744724	SAMN20115097	JAHVWM000000000	SRR17225271	Farm 5	feed	January 2020	Oppland
D077L	dairy farm	yes	ST-18	CC18	II	PRJNA744724	SAMN20115098	JAHVWN000000000	SRR17225270	Farm 1	feces	January 2020	Oppland
D078L	dairy farm	yes	ST-394	CC415	II	PRJNA744724	SAMN20115099	JAHVWO000000000	SRR17225268	Farm 2	feed	January 2020	Oppland
D080L	dairy farm	yes	ST-425	CC90	II	PRJNA744724	SAMN20115100	JAHVWP000000000	SRR17225267	Farm 1	feed	January 2020	Oppland
D081L	dairy farm	yes	ST-2761	CC18	II	PRJNA744724	SAMN20115101	JAHVWQ000000000	SRR17225266	Farm 3	feces	January 2020	Oppland
D084L	dairy farm	yes	ST-224	CC224	I	PRJNA744724	SAMN20115102	JAHVWR000000000	SRR17225265	Farm 9	feed	January 2020	Akershus
D087L	dairy farm	yes	ST-4	CC4	I	PRJNA744724	SAMN20115103	JAHVWS000000000	SRR17225264	Farm 11	feed	January 2020	Østfold
D091L	dairy farm	yes	ST-37	CC37	II	PRJNA744724	SAMN20115104	JAHVWT000000000	SRR17225263	Farm 12	milk filter	January 2020	Østfold
D092L	dairy farm	yes	ST-451	CC11	II	PRJNA744724	SAMN20115105	JAHVWU000000000	SRR17225262	Farm 12	feed	January 2020	Østfold
D102L	dairy farm	yes	ST-91	CC91	II	PRJNA744724	SAMN20115106	JAHVWV000000000	SRR17225261	Farm 15	feed	January 2020	Buskerud
D103L	dairy farm	yes	ST-18	CC18	II	PRJNA744724	SAMN20115107	JAHVWW000000000	SRR17225260	Farm 17	feces	January 2020	Buskerud
D106L	dairy farm	yes	ST-91	CC91	II	PRJNA744724	SAMN20115108	JAHVWX000000000	SRR17225259	Farm 14	feed	January 2020	Buskerud
D107L	dairy farm	yes	ST-394	CC415	II	PRJNA744724	SAMN20115109	JAHVWY000000000	SRR17225257	Farm 2	feed	February 2020	Oppland
D108L	dairy farm	yes	ST-2761	CC18	II	PRJNA744724	SAMN20115110	JAHVWZ000000000	SRR17225256	Farm 3	feces	February 2020	Oppland
D115L	dairy farm	yes	ST-37	CC37	II	PRJNA744724	SAMN20115111	JAHVXA000000000	SRR17225255	Farm 1	feed	February 2020	Oppland
D116L	dairy farm	yes	ST-20	CC20	II	PRJNA744724	SAMN20115112	JAHVXB000000000	SRR17225254	Farm 1	teat swab	February 2020	Oppland
D117L	dairy farm	yes	ST-451	CC11	II	PRJNA744724	SAMN20115113	JAHVXC000000000	SRR17225253	Farm 6	feces	February 2020	Akershus
D118L	dairy farm	yes	ST-451	CC11	II	PRJNA744724	SAMN20115114	JAHVXD000000000	SRR17225252	Farm 6	feed	February 2020	Akershus
D129L	dairy farm	yes	ST-21	CC21	II	PRJNA744724	SAMN20115115	JAHVXE000000000	SRR17225251	Farm 10	feces	February 2020	Østfold
D130L	dairy farm	yes	ST-451	CC11	II	PRJNA744724	SAMN20115116	JAHVXF000000000	SRR17225250	Farm 11	feces	February 2020	Østfold
D135L	dairy farm	yes	ST-412	CC412	II	PRJNA744724	SAMN20115117	JAHVXG000000000	SRR17225249	Farm 10	feed	February 2020	Østfold
D136L	dairy farm	yes	ST-177	CC177	II	PRJNA744724	SAMN20115118	JAHVXH000000000	SRR17225248	Farm 12	feed	March 2020	Østfold
D137L	dairy farm	yes	ST-91	CC91	II	PRJNA744724	SAMN20115119	JAHVCD000000000	SRR17225246	Farm 14	feces	March 2020	Buskerud
D138L	dairy farm	yes	ST-177	CC177	II	PRJNA744724	SAMN20115120	JAHVCC000000000	SRR17225245	Farm 12	milk filter	March 2020	Østfold
D139L	dairy farm	yes	ST-177	CC177	II	PRJNA744724	SAMN20115121	JAHVCB000000000	SRR17225244	Farm 12	feces	March 2020	Østfold

D140L	dairy farm	yes	ST-91	CC91	II	PRJNA744724	SAMN20115122	JAHVCA000000000	SRR17225243	Farm 14	feed	March 2020	Buskerud
D141L	dairy farm	yes	ST-18	CC18	II	PRJNA744724	SAMN20115123	JAHVBZ000000000	SRR17225242	Farm 1	milk filter	January 2020	Oppland
D142L	dairy farm	yes	ST-20	CC20	II	PRJNA744724	SAMN20115124	JAHVBY000000000	SRR17225241	Farm 1	feces	February 2020	Oppland
D143L	dairy farm	yes	ST-2761	CC18	II	PRJNA744724	SAMN20115125	JAHVBX000000000	SRR17225240	Farm 3	feed	January 2020	Oppland
D144L	dairy farm	yes	ST-11	CC11	II	PRJNA744724	SAMN20115126	JAHVBW000000000	SRR17225239	Farm 10	feces	September 2019	Østfold
D145L	dairy farm	yes	ST-451	CC11	II	PRJNA744724	SAMN20115127	JAHVBV000000000	SRR17225238	Farm 12	feces	January 2020	Østfold
D146L	dairy farm	yes	ST-18	CC18	II	PRJNA744724	SAMN20115128	JAHVBU000000000	SRR17225237	Farm 17	feed	January 2020	Buskerud
D158L	dairy farm	yes	ST-91	CC91	II	PRJNA744724	SAMN20115129	JAHVBT000000000	SRR17225235	Farm 12	milk filter	May 2020	Østfold
D159L	dairy farm	yes	ST-37	CC37	II	PRJNA744724	SAMN20115130	JAHVBS000000000	SRR17225234	Farm 12	teat swab	May 2020	Østfold
D161L	dairy farm	yes	ST-8	CC8	II	PRJNA744724	SAMN20115132	JAHVBQ000000000	SRR17225232	Farm 13	feed	May 2020	Østfold
D162L	dairy farm	yes	ST-91	CC91	II	PRJNA744724	SAMN20115133	JAHVBP000000000	SRR17225231	Farm 13	teat swab	May 2020	Østfold
D163L	dairy farm	yes	ST-511	CC7	II	PRJNA744724	SAMN20115134	JAHVBO000000000	SRR17225230	Farm 12	feed	May 2020	Østfold
D164L	dairy farm	yes	ST-37	CC37	II	PRJNA744724	SAMN20115135	JAHVBN000000000	SRR17225229	Farm 13	feces	May 2020	Østfold
D169L	dairy farm	yes	ST-91	CC91	II	PRJNA744724	SAMN20115136	JAHVBM000000000	SRR17225228	Farm 12	feces	May 2020	Østfold
D170L	dairy farm	yes	ST-91	CC91	II	PRJNA744724	SAMN20115137	JAHVBL000000000	SRR17225227	Farm 18	feed	May 2020	Buskerud
D181L	dairy farm	yes	ST-37	CC37	II	PRJNA744724	SAMN20115138	JAHVBK000000000	SRR17225226	Farm 5	feces	June 2020	Oppland
D182L	dairy farm	yes	ST-4	CC4	I	PRJNA744724	SAMN20115139	JAHVBJ000000000	SRR17225224	Farm 11	feces	June 2020	Østfold
D189L	dairy farm	yes	ST-412	CC412	II	PRJNA744724	SAMN20115140	JAHVBI000000000	SRR17225223	Farm 10	feces	June 2020	Østfold
D190L	dairy farm	yes	ST-124	CC124	II	PRJNA744724	SAMN20115141	JAHVBH000000000	SRR17225222	Farm 13	feces	June 2020	Østfold
D191L	dairy farm	yes	ST-451	CC11	II	PRJNA744724	SAMN20115142	JAHVBG000000000	SRR17225221	Farm 12	feces	June 2020	Østfold
D192L	dairy farm	yes	ST-177	CC177	II	PRJNA744724	SAMN20115143	JAHVBF000000000	SRR17225220	Farm 12	feed	June 2020	Østfold
D199L	dairy farm	yes	ST-91	CC91	II	PRJNA744724	SAMN20115144	JAHVBE000000000	SRR17225219	Farm 6	milk filter	June 2020	Akershus
D200L	dairy farm	yes	ST-451	CC11	II	PRJNA744724	SAMN20115145	JAHVBD000000000	SRR17225218	Farm 8	milk filter	June 2020	Akershus
D201L	dairy farm	yes	ST-451	CC11	II	PRJNA744724	SAMN20115146	JAHVBC000000000	SRR17225217	Farm 9	milk filter	June 2020	Akershus
D202L	dairy farm	yes	ST-21	CC21	II	PRJNA744724	SAMN20115147	JAHVBB000000000	SRR17225216	Farm 9	feces	June 2020	Akershus
D203L	dairy farm	yes	ST-451	CC11	II	PRJNA744724	SAMN20115148	JAHVBA000000000	SRR17225215	Farm 9	feed	June 2020	Akershus
D204L	dairy farm	yes	ST-91	CC91	II	PRJNA744724	SAMN20115149	JAHVAZ000000000	SRR17225213	Farm 13	milk filter	May 2020	Østfold
D205L	dairy farm	yes	ST-394	CC415	II	PRJNA744724	SAMN20115150	JAHVAY000000000	SRR17225212	Farm 10	feed	June 2020	Østfold
ERR2522241	clinical	no	ST-31	CC31	II	PRJEB26061	SAMEA4586991	-	ERR2522241			2010	Norway
ERR2522242	clinical	yes	ST-20	CC20	II	PRJEB26061	SAMEA4586992	-	ERR2522242			2010	Norway
ERR2522243	clinical	yes	ST-8	CC8	II	PRJEB26061	SAMEA4586993	-	ERR2522243			2010	Norway
ERR2522244	clinical	yes	ST-87	CC87	I	PRJEB26061	SAMEA4586994	-	ERR2522244			2010	Norway
ERR2522245	clinical	no	ST-1	CC1	I	PRJEB26061	SAMEA4586995	-	ERR2522245			2010	Norway
ERR2522246	clinical	no	ST-20	CC20	II	PRJEB26061	SAMEA4586996	-	ERR2522246			2010	Norway
ERR2522247	clinical	yes	ST-7	CC7	II	PRJEB26061	SAMEA4586997	-	ERR2522247			2010	Norway
ERR2522248	clinical	no	ST-7	CC7	II	PRJEB26061	SAMEA4586998	-	ERR2522248			2010	Norway
ERR2522249	clinical	yes	ST-101	CC101	II	PRJEB26061	SAMEA4586999	-	ERR2522249			2010	Norway
ERR2522250	clinical	no	ST-101	CC101	II	PRJEB26061	SAMEA4587000	-	ERR2522250			2010	Norway
ERR2522251	clinical	yes	ST-199	CC199	II	PRJEB26061	SAMEA4587001	-	ERR2522251			2010	Norway
ERR2522252	clinical	no	ST-7	CC7	II	PRJEB26061	SAMEA4587002	-	ERR2522252			2010	Norway
ERR2522253	clinical	yes	ST-7	CC7	II	PRJEB26061	SAMEA4587003	-	ERR2522253			2010	Norway
ERR2522254	clinical	no	ST-1	CC1	I	PRJEB26061	SAMEA4587004	-	ERR2522254			2010	Norway
ERR2522255	clinical	yes	ST-101	CC101	II	PRJEB26061	SAMEA4587005	-	ERR2522255			2010	Norway
ERR2522256	clinical	no	ST-399	CC14	II	PRJEB26061	SAMEA4587006	-	ERR2522256			2010	Norway
ERR2522257	clinical	yes	ST-7	CC7	II	PRJEB26061	SAMEA4587007	-	ERR2522257			2010	Norway
ERR2522258	clinical	yes	ST-121	CC121	II	PRJEB26061	SAMEA4587008	-	ERR2522258			2010	Norway
ERR2522259	clinical	yes	ST-18	CC18	II	PRJEB26061	SAMEA4587009	-	ERR2522259			2010	Norway
ERR2522260	clinical	no	ST-399	CC14	II	PRJEB26061	SAMEA4587010	-	ERR2522260			2010	Norway
ERR2522261	clinical	yes	ST-399	CC14	II	PRJEB26061	SAMEA4587011	-	ERR2522261			2010	Norway
ERR2522262	clinical	no	ST-7	CC7	II	PRJEB26061	SAMEA4587012	-	ERR2522262			2011	Norway
ERR2522263	clinical	no	ST-121	CC121	II	PRJEB26061	SAMEA4587013	-	ERR2522263			2011	Norway
ERR2522264	clinical	no	unknown	unknown	unknown	PRJEB26061	SAMEA4587014	-	ERR2522264			2011	Norway
ERR2522265	clinical	yes	ST-394	CC415	II	PRJEB26061	SAMEA4587015	-	ERR2522265			2011	Norway
ERR2522266	clinical	yes	ST-7	CC7	II	PRJEB26061	SAMEA4587016	-	ERR2522266			2011	Norway
ERR2522267	clinical	no	ST-180	CC177	II	PRJEB26061	SAMEA4587017	-	ERR2522267			2011	Norway
ERR2522268	clinical	no	ST-399	CC14	II	PRJEB26061	SAMEA4587018	-	ERR2522268			2011	Norway
ERR2522269	clinical	yes	ST-394	CC415	II	PRJEB26061	SAMEA4587019	-	ERR2522269			2011	Norway

ERR2522270	clinical	yes	ST-325	CC31	II	PRJEB26061	SAMEA4587020	-	ERR2522270			2011	Norway
ERR2522271	clinical	no	ST-8	CC8	II	PRJEB26061	SAMEA4587021	-	ERR2522271			2011	Norway
ERR2522272	clinical	no	ST-91	CC91	II	PRJEB26061	SAMEA4587022	-	ERR2522272			2011	Norway
ERR2522273	clinical	no	ST-7	CC7	II	PRJEB26061	SAMEA4587023	-	ERR2522273			2011	Norway
ERR2522274	clinical	no	ST-9	CC9	II	PRJEB26061	SAMEA4587024	-	ERR2522274			2011	Norway
ERR2522275	clinical	no	ST-8	CC8	II	PRJEB26061	SAMEA4587025	-	ERR2522275			2011	Norway
ERR2522276	clinical	no	ST-121	CC121	II	PRJEB26061	SAMEA4587026	-	ERR2522276			2011	Norway
ERR2522277	clinical	yes	ST-7	CC7	II	PRJEB26061	SAMEA4587027	-	ERR2522277			2011	Norway
ERR2522278	clinical	no	ST-8	CC8	II	PRJEB26061	SAMEA4587028	-	ERR2522278			2011	Norway
ERR2522279	clinical	yes	ST-14	CC14	II	PRJEB26061	SAMEA4587029	-	ERR2522279			2011	Norway
ERR2522280	clinical	yes	ST-121	CC121	II	PRJEB26061	SAMEA4587030	-	ERR2522280			2011	Norway
ERR2522281	clinical	yes	ST-87	CC87	I	PRJEB26061	SAMEA4587031	-	ERR2522281			2011	Norway
ERR2522282	clinical	yes	ST-7	CC7	II	PRJEB26061	SAMEA4587032	-	ERR2522282			2012	Norway
ERR2522283	clinical	yes	ST-14	CC14	II	PRJEB26061	SAMEA4587033	-	ERR2522283			2012	Norway
ERR2522284	clinical	yes	ST-121	CC121	II	PRJEB26061	SAMEA4587034	-	ERR2522284			2012	Norway
ERR2522285	clinical	yes	ST-59	CC59	I	PRJEB26061	SAMEA4587035	-	ERR2522285			2012	Norway
ERR2522286	clinical	yes	ST-8	CC8	II	PRJEB26061	SAMEA4587036	-	ERR2522286			2012	Norway
ERR2522287	clinical	yes	ST-7	CC7	II	PRJEB26061	SAMEA4587037	-	ERR2522287			2012	Norway
ERR2522288	clinical	yes	ST-7	CC7	II	PRJEB26061	SAMEA4587038	-	ERR2522288			2012	Norway
ERR2522289	clinical	yes	ST-9	CC9	II	PRJEB26061	SAMEA4587039	-	ERR2522289			2012	Norway
ERR2522290	clinical	yes	ST-7	CC7	II	PRJEB26061	SAMEA4587040	-	ERR2522290			2012	Norway
ERR2522291	clinical	yes	ST-219	CC4	I	PRJEB26061	SAMEA4587041	-	ERR2522291			2012	Norway
ERR2522292	clinical	yes	ST-121	CC121	II	PRJEB26061	SAMEA4587042	-	ERR2522292			2012	Norway
ERR2522293	clinical	yes	ST-1	CC1	I	PRJEB26061	SAMEA4587043	-	ERR2522293			2012	Norway
ERR2522294	clinical	yes	ST-121	CC121	II	PRJEB26061	SAMEA4587044	-	ERR2522294			2012	Norway
ERR2522295	clinical	yes	ST-121	CC121	II	PRJEB26061	SAMEA4587045	-	ERR2522295			2012	Norway
ERR2522296	clinical	yes	ST-20	CC20	II	PRJEB26061	SAMEA4587046	-	ERR2522296			2012	Norway
ERR2522297	clinical	yes	ST-121	CC121	II	PRJEB26061	SAMEA4587047	-	ERR2522297			2012	Norway
ERR2522298	clinical	yes	ST-391	CC89	II	PRJEB26061	SAMEA4587048	-	ERR2522298			2012	Norway
ERR2522299	clinical	yes	ST-7	CC7	II	PRJEB26061	SAMEA4587049	-	ERR2522299			2012	Norway
ERR2522300	clinical	yes	ST-87	CC87	I	PRJEB26061	SAMEA4587050	-	ERR2522300			2012	Norway
ERR2522301	clinical	yes	ST-6	CC6	I	PRJEB26061	SAMEA4587051	-	ERR2522301			2012	Norway
ERR2522302	clinical	yes	ST-7	CC7	II	PRJEB26061	SAMEA4587052	-	ERR2522302			2012	Norway
ERR2522303	clinical	yes	ST-7	CC7	II	PRJEB26061	SAMEA4587053	-	ERR2522303			2012	Norway
ERR2522304	clinical	yes	ST-220	CC220	I	PRJEB26061	SAMEA4587054	-	ERR2522304			2012	Norway
ERR2522305	clinical	yes	ST-7	CC7	II	PRJEB26061	SAMEA4587055	-	ERR2522305			2012	Norway
ERR2522306	clinical	yes	ST-20	CC20	II	PRJEB26061	SAMEA4587056	-	ERR2522306			2012	Norway
ERR2522307	clinical	yes	ST-20	CC20	II	PRJEB26061	SAMEA4587057	-	ERR2522307			2012	Norway
ERR2522308	clinical	yes	ST-177	CC177	II	PRJEB26061	SAMEA4587058	-	ERR2522308			2012	Norway
ERR2522309	clinical	yes	ST-1	CC1	I	PRJEB26061	SAMEA4587059	-	ERR2522309			2013	Norway
ERR2522310	clinical	yes	ST-691	CC7	II	PRJEB26061	SAMEA4587060	-	ERR2522310			2013	Norway
ERR2522311	clinical	yes	ST-1416	CC19	II	PRJEB26061	SAMEA4587061	-	ERR2522311			2013	Norway
ERR2522312	clinical	yes	ST-121	CC121	II	PRJEB26061	SAMEA4587062	-	ERR2522312			2013	Norway
ERR2522313	clinical	yes	ST-87	CC87	I	PRJEB26061	SAMEA4587063	-	ERR2522313			2013	Norway
ERR2522314	clinical	yes	ST-451	CC11	II	PRJEB26061	SAMEA4587064	-	ERR2522314			2013	Norway
ERR2522315	clinical	yes	ST-37	CC37	II	PRJEB26061	SAMEA4587065	-	ERR2522315			2013	Norway
ERR2522316	clinical	yes	ST-1	CC1	I	PRJEB26061	SAMEA4587066	-	ERR2522316			2013	Norway
ERR2522317	clinical	yes	ST-1	CC1	I	PRJEB26061	SAMEA4587067	-	ERR2522317			2013	Norway
ERR2522318	clinical	yes	ST-802	CC19	II	PRJEB26061	SAMEA4587068	-	ERR2522318			2013	Norway
ERR2522319	clinical	yes	ST-8	CC8	II	PRJEB26061	SAMEA4587069	-	ERR2522319			2013	Norway
ERR2522320	clinical	yes	ST-802	CC19	II	PRJEB26061	SAMEA4587070	-	ERR2522320			2013	Norway
ERR2522321	clinical	yes	ST-802	CC19	II	PRJEB26061	SAMEA4587071	-	ERR2522321			2013	Norway
ERR2522322	clinical	yes	ST-220	CC220	I	PRJEB26061	SAMEA4587072	-	ERR2522322			2013	Norway
ERR2522323	clinical	yes	ST-451	CC11	II	PRJEB26061	SAMEA4587073	-	ERR2522323			2013	Norway
ERR2522324	clinical	yes	ST-802	CC19	II	PRJEB26061	SAMEA4587074	-	ERR2522324			2013	Norway
ERR2522325	clinical	yes	ST-8	CC8	II	PRJEB26061	SAMEA4587075	-	ERR2522325			2013	Norway
ERR2522326	clinical	yes	ST-1	CC1	I	PRJEB26061	SAMEA4587076	-	ERR2522326			2013	Norway

ERR2522327	clinical	yes	ST-177	CC177	II	PRJEB26061	SAMEA4587077	-	ERR2522327			2014	Norway
ERR2522328	clinical	yes	ST-7	CC7	II	PRJEB26061	SAMEA4587078	-	ERR2522328			2014	Norway
ERR2522329	clinical	yes	ST-7	CC7	II	PRJEB26061	SAMEA4587079	-	ERR2522329			2014	Norway
ERR2522330	clinical	yes	ST-7	CC7	II	PRJEB26061	SAMEA4587080	-	ERR2522330			2014	Norway
ERR2522331	clinical	yes	ST-7	CC7	II	PRJEB26061	SAMEA4587081	-	ERR2522331			2014	Norway
ERR2522332	clinical	yes	ST-18	CC18	II	PRJEB26061	SAMEA4587082	-	ERR2522332			2014	Norway
ERR2522333	clinical	yes	ST-8	CC8	II	PRJEB26061	SAMEA4587083	-	ERR2522333			2014	Norway
ERR2522334	clinical	yes	ST-7	CC7	II	PRJEB26061	SAMEA4587084	-	ERR2522334			2014	Norway
ERR2522335	clinical	yes	ST-121	CC121	II	PRJEB26061	SAMEA4587085	-	ERR2522335			2014	Norway
ERR2522336	clinical	yes	ST-5	CC5	I	PRJEB26061	SAMEA4587086	-	ERR2522336			2014	Norway
ERR2522337	clinical	yes	ST-121	CC121	II	PRJEB26061	SAMEA4587087	-	ERR2522337			2014	Norway
ERR2522338	clinical	yes	ST-121	CC121	II	PRJEB26061	SAMEA4587088	-	ERR2522338			2014	Norway
ERR2522339	clinical	yes	ST-6	CC6	I	PRJEB26061	SAMEA4587089	-	ERR2522339			2014	Norway
ERR2522340	clinical	yes	ST-121	CC121	II	PRJEB26061	SAMEA4587090	-	ERR2522340			2014	Norway
ERR2522341	clinical	yes	ST-18	CC18	II	PRJEB26061	SAMEA4587091	-	ERR2522341			2014	Norway
ERR2522342	clinical	yes	ST-4	CC4	I	PRJEB26061	SAMEA4587092	-	ERR2522342			2014	Norway
ERR2522343	clinical	yes	ST-7	CC7	II	PRJEB26061	SAMEA4587093	-	ERR2522343			2014	Norway
ERR2522344	clinical	yes	ST-91	CC91	II	PRJEB26061	SAMEA4587094	-	ERR2522344			2014	Norway
ERR2522345	clinical	yes	ST-7	CC7	II	PRJEB26061	SAMEA4587095	-	ERR2522345			2014	Norway
ERR2522346	clinical	yes	ST-8	CC8	II	PRJEB26061	SAMEA4587096	-	ERR2522346			2014	Norway
ERR2522347	clinical	yes	ST-7	CC7	II	PRJEB26061	SAMEA4587097	-	ERR2522347			2014	Norway
ERR2522348	clinical	yes	ST-403	CC403	II	PRJEB26061	SAMEA4587098	-	ERR2522348			2014	Norway
ERR2522349	clinical	yes	ST-226	CC226	II	PRJEB26061	SAMEA4587099	-	ERR2522349			2014	Norway
ERR2522350	clinical	yes	ST-5	CC5	I	PRJEB26061	SAMEA4587100	-	ERR2522350			2014	Norway
ERR2522351	clinical	yes	ST-7	CC7	II	PRJEB26061	SAMEA4587101	-	ERR2522351			2014	Norway
ERR2522352	clinical	yes	ST-121	CC121	II	PRJEB26061	SAMEA4587102	-	ERR2522352			2014	Norway
ERR2522353	clinical	yes	ST-121	CC121	II	PRJEB26061	SAMEA4587103	-	ERR2522353			2015	Norway
ERR2522354	clinical	yes	ST-296	CC88	I	PRJEB26061	SAMEA4587104	-	ERR2522354			2015	Norway
ERR2522355	clinical	yes	ST-7	CC7	II	PRJEB26061	SAMEA4587105	-	ERR2522355			2015	Norway
ERR2522356	clinical	yes	ST-18	CC18	II	PRJEB26061	SAMEA4587106	-	ERR2522356			2015	Norway
ERR2522357	clinical	yes	ST-18	CC18	II	PRJEB26061	SAMEA4587107	-	ERR2522357			2015	Norway
ERR2522358	clinical	yes	ST-296	CC88	I	PRJEB26061	SAMEA4587108	-	ERR2522358			2015	Norway
ERR2522359	clinical	yes	ST-7	CC7	II	PRJEB26061	SAMEA4587109	-	ERR2522359			2015	Norway
ERR2522360	clinical	yes	ST-7	CC7	II	PRJEB26061	SAMEA4587110	-	ERR2522360			2015	Norway
ERR2522361	clinical	yes	ST-226	CC226	II	PRJEB26061	SAMEA4587111	-	ERR2522361			2015	Norway
ERR2522362	clinical	yes	ST-8	CC8	II	PRJEB26061	SAMEA4587112	-	ERR2522362			2015	Norway
ERR2522363	clinical	yes	ST-121	CC121	II	PRJEB26061	SAMEA4587113	-	ERR2522363			2015	Norway
ERR2522364	clinical	yes	ST-87	CC87	I	PRJEB26061	SAMEA4587114	-	ERR2522364			2015	Norway
ERR2522365	clinical	yes	ST-7	CC7	II	PRJEB26061	SAMEA4587115	-	ERR2522365			2015	Norway
ERR2522366	clinical	yes	ST-87	CC87	I	PRJEB26061	SAMEA4587116	-	ERR2522366			2015	Norway
ERR2522367	clinical	yes	ST-3	CC3	I	PRJEB26061	SAMEA4587117	-	ERR2522367			2015	Norway
ERR2522368	clinical	yes	ST-37	CC37	II	PRJEB26061	SAMEA4587118	-	ERR2522368			2015	Norway
ERR2522369	clinical	yes	ST-9	CC9	II	PRJEB26061	SAMEA4587119	-	ERR2522369			2015	Norway
ERR2522370	clinical	yes	ST-1	CC1	I	PRJEB26061	SAMEA4587120	-	ERR2522370			2015	Norway
ERR3047199	clinical	yes	ST-20	CC20	II	PRJEB25848	SAMEA5209053	-	ERR3047199			2018	Norway
ERR3446056	clinical	yes	ST-37	CC37	II	PRJEB25848	SAMEA5801351	-	ERR3446056			2018	Norway
MF6549	rural/urban	yes	ST-451	CC11	II	PRJNA689486	SAMN17211659	JAEPBE000000000	SRR13590815	33	Urban or residential area	October 2016	Akershus
MF6550	rural/urban	yes	ST-451	CC11	II	PRJNA689486	SAMN17211660	JAEPBD000000000	SRR13590814	48	Urban or residential area	October 2016	Akershus
MF6551	rural/urban	yes	ST-451	CC11	II	PRJNA689486	SAMN17211661	JAEPBC000000000	SRR13590786	49	Urban or residential area	October 2016	Akershus
MF6552	rural/urban	yes	ST-451	CC11	II	PRJNA689486	SAMN17211662	JAEPBB000000000	SRR13590775	50	Urban or residential area	October 2016	Akershus
MF6554	rural/urban	yes	ST-4	CC4	I	PRJNA689486	SAMN17211664	JAEPAZ000000000	SRR13590753	53	Grazeland or animal path	October 2016	Akershus
MF6555	rural/urban	yes	ST-91	CC91	II	PRJNA689486	SAMN17211665	JAEPAY000000000	SRR13590742	54	Grazeland or animal path	October 2016	Akershus
MF6556	rural/urban	yes	ST-37	CC37	II	PRJNA689486	SAMN17211666	JAEPAX000000000	SRR13590731	55	Grazeland or animal path	October 2016	Akershus
MF6557	rural/urban	yes	ST-91	CC91	II	PRJNA689486	SAMN17211667	JAEPAW000000000	SRR13590720	56	Grazeland or animal path	October 2016	Akershus
MF6558	rural/urban	yes	ST-20	CC20	II	PRJNA689486	SAMN17211668	JAEPAV000000000	SRR13590709	66	Urban or residential area	October 2016	Akershus
MF6711	rural/urban	yes	ST-398	CC19	II	PRJNA689486	SAMN17211669	JAEPAU000000000	SRR13590813	98	Grazeland or animal path	June 2017	Akershus
MF6712	rural/urban	yes	ST-398	CC19	II	PRJNA689486	SAMN17211670	JAEPAT000000000	SRR13590802	99	Grazeland or animal path	June 2017	Akershus

MF6713	rural/urban	yes	ST-398	CC19	II	PRJNA689486	SAMN17211671	JAEPAS000000000	SRR13590794	100	Grazeland or animal path	June 2017	Akershus
MF6714	rural/urban	yes	ST-398	CC19	II	PRJNA689486	SAMN17211672	JAEPAR000000000	SRR13590793	101	Grazeland or animal path	June 2017	Akershus
MF6716	rural/urban	yes	ST-4	CC4	I	PRJNA689486	SAMN17211673	JAEPAQ000000000	SRR13590792	109	Grazeland or animal path	June 2017	Akershus
MF6717	rural/urban	yes	ST-91	CC91	II	PRJNA689486	SAMN17211674	JAEPAP000000000	SRR13590791	113	Forest or mountain area	June 2017	Akershus
MF6718	rural/urban	yes	ST-37	CC37	II	PRJNA689486	SAMN17211675	JAEPAO000000000	SRR13590790	115	Grazeland or animal path	June 2017	Akershus
MF6719	rural/urban	yes	ST-398	CC19	II	PRJNA689486	SAMN17211676	JAEPAN000000000	SRR13590789	118	Urban or residential area	June 2017	Akershus
MF6720	rural/urban	yes	ST-91	CC91	II	PRJNA689486	SAMN17211677	JAEPAM000000000	SRR13590788	120	Urban or residential area	June 2017	Akershus
MF6721	rural/urban	yes	ST-399	CC14	II	PRJNA689486	SAMN17211678	JAEPJO000000000	SRR13590787	121	Urban or residential area	June 2017	Akershus
MF6722	rural/urban	yes	ST-398	CC19	II	PRJNA689486	SAMN17211679	JAEPJN000000000	SRR13590785	123	Urban or residential area	June 2017	Akershus
MF6723	rural/urban	yes	ST-37	CC37	II	PRJNA689486	SAMN17211680	JAEPAL000000000	SRR13590784	129	Urban or residential area	June 2017	Akershus
MF6724	rural/urban	yes	ST-91	CC91	II	PRJNA689486	SAMN17211681	JAEPAK000000000	SRR13590783	130	Grazeland or animal path	June 2017	Akershus
MF6725	rural/urban	yes	ST-29	CC29	II	PRJNA689486	SAMN17211682	JAEPAJ000000000	SRR13590782	131	Grazeland or animal path	June 2017	Akershus
MF6726	rural/urban	yes	ST-91	CC91	II	PRJNA689486	SAMN17211683	JAEPAI000000000	SRR13590781	132	Grazeland or animal path	June 2017	Akershus
MF6727	rural/urban	yes	ST-398	CC19	II	PRJNA689486	SAMN17211684	JAEPAH000000000	SRR13590780	133	Grazeland or animal path	June 2017	Akershus
MF6728	rural/urban	yes	ST-29	CC29	II	PRJNA689486	SAMN17211685	JAEPAG000000000	SRR13590779	134	Grazeland or animal path	June 2017	Akershus
MF6804	rural/urban	yes	ST-451	CC11	II	PRJNA689486	SAMN17211686	JAEPAF000000000	SRR13590778	183	Grazeland or animal path	September 2017	Troms
MF6805	rural/urban	yes	ST-18	CC18	II	PRJNA689486	SAMN17211687	JAEPJM000000000	SRR13590777	184	Grazeland or animal path	September 2017	Troms
MF6806	rural/urban	yes	ST-18	CC18	II	PRJNA689486	SAMN17211688	JAEPAE000000000	SRR13590776	185	Grazeland or animal path	September 2017	Troms
MF6807	rural/urban	yes	ST-18	CC18	II	PRJNA689486	SAMN17211689	JAEPAD000000000	SRR13590774	188	Agricultural field	September 2017	Troms
MF6825	rural/urban	yes	ST-37	CC37	II	PRJNA689486	SAMN17211690	JAEPAC000000000	SRR13590773	248	Urban or residential area	October 2017	Oslo
MF6826	rural/urban	yes	ST-18	CC18	II	PRJNA689486	SAMN17211691	JAEPAB000000000	SRR13590772	249	Urban or residential area	October 2017	Oslo
MF6827	rural/urban	yes	ST-18	CC18	II	PRJNA689486	SAMN17211692	JAEPAA000000000	SRR13590771	251	Urban or residential area	October 2017	Oslo
MF6828	rural/urban	yes	ST-204	CC204	II	PRJNA689486	SAMN17211693	JAEOZZ000000000	SRR13590770	252	Urban or residential area	October 2017	Oslo
MF6829	rural/urban	yes	ST-1	CC1	I	PRJNA689486	SAMN17211694	JAEPJL000000000	SRR13590769	253	Urban or residential area	October 2017	Oslo
MF6830	rural/urban	yes	ST-204	CC204	II	PRJNA689486	SAMN17211695	JAEOZY000000000	SRR13590768	259	Urban or residential area	October 2017	Oslo
MF6831	rural/urban	yes	ST-6	CC6	I	PRJNA689486	SAMN17211696	JAEOZX000000000	SRR13590767	262	Urban or residential area	October 2017	Oslo
MF6832	rural/urban	yes	ST-91	CC91	II	PRJNA689486	SAMN17211697	JAEOZW000000000	SRR13590766	267	Urban or residential area	October 2017	Oslo
MF6833	rural/urban	yes	ST-6	CC6	I	PRJNA689486	SAMN17211698	JAEOZV000000000	SRR13590765	268	Urban or residential area	October 2017	Oslo
MF6834	rural/urban	yes	ST-403	CC403	II	PRJNA689486	SAMN17211699	JAEOZU000000000	SRR13590763	269	Urban or residential area	October 2017	Oslo
MF6835	rural/urban	yes	ST-8	CC8	II	PRJNA689486	SAMN17211700	JAEOZT000000000	SRR13590762	246	Urban or residential area	October 2017	Oslo
MF6838	rural/urban	yes	ST-732	CC7	II	PRJNA689486	SAMN17211701	JAEPJK000000000	SRR13590761	279	Near food processing plant	November 2017	Sogn og Fjordane
MF6839	rural/urban	yes	ST-325	CC31	II	PRJNA689486	SAMN17211702	JAEOZS000000000	SRR13590760	285	Near food processing plant	November 2017	Sogn og Fjordane
MF6840	rural/urban	yes	ST-1	CC1	I	PRJNA689486	SAMN17211703	JAEOZR000000000	SRR13590759	287	Near food processing plant	November 2017	Sogn og Fjordane
MF6841	rural/urban	yes	ST-504	CC475	II	PRJNA689486	SAMN17211704	JAEOZQ000000000	SRR13590758	288	Near food processing plant	November 2017	Sogn og Fjordane
MF6983	rural/urban	yes	ST-1	CC1	I	PRJNA689486	SAMN17211705	JAEOZP000000000	SRR13590757	293	Urban or residential area	May 2018	Østfold
MF6984	rural/urban	yes	ST-91	CC91	II	PRJNA689486	SAMN17211706	JAEOZO000000000	SRR13590756	298	Urban or residential area	May 2018	Østfold
MF6985	rural/urban	yes	ST-20	CC20	II	PRJNA689486	SAMN17211707	JAEOZN000000000	SRR13590755	323	Urban or residential area	June 2018	Troms
MF6986	rural/urban	yes	ST-20	CC20	II	PRJNA689486	SAMN17211708	JAEOZM000000000	SRR13590754	358	Near food processing plant	June 2018	Sogn og Fjordane
MF6988	rural/urban	yes	ST-732	CC7	II	PRJNA689486	SAMN17211709	JAEOZL000000000	SRR13590752	279	Near food processing plant	June 2018	Sogn og Fjordane
MF7046	rural/urban	yes	ST-732	CC7	II	PRJNA689486	SAMN17211710	JAEPJJ000000000	SRR13590751	279	Near food processing plant	September 2018	Sogn og Fjordane
MF7047	rural/urban	yes	ST-37	CC37	II	PRJNA689486	SAMN17211711	JAEPJI000000000	SRR13590750	365	Near food processing plant	September 2018	Sogn og Fjordane
MF7048	rural/urban	yes	ST-647	CC20	II	PRJNA689486	SAMN17211712	JAEOZK000000000	SRR13590749	287	Near food processing plant	September 2018	Sogn og Fjordane
MF7049	rural/urban	yes	ST-647	CC20	II	PRJNA689486	SAMN17211713	JAEOZJ000000000	SRR13590748	354	Near food processing plant	September 2018	Sogn og Fjordane
MF7051	rural/urban	yes	ST-732	CC7	II	PRJNA689486	SAMN17211715	JAEOZI000000000	SRR13590746	390	Near food processing plant	September 2018	Sogn og Fjordane
MF7052	rural/urban	yes	ST-732	CC7	II	PRJNA689486	SAMN17211716	JAEOZH000000000	SRR13590745	391	Near food processing plant	September 2018	Sogn og Fjordane
MF7053	rural/urban	yes	ST-732	CC7	II	PRJNA689486	SAMN17211717	JAEPJG000000000	SRR13590744	394	Near food processing plant	September 2018	Sogn og Fjordane
MF7054	rural/urban	yes	ST-732	CC7	II	PRJNA689486	SAMN17211718	JAEPJF000000000	SRR13590743	363	Near food processing plant	September 2018	Sogn og Fjordane
MF7055	rural/urban	yes	ST-732	CC7	II	PRJNA689486	SAMN17211719	JAEPJE000000000	SRR13590741	375	Near food processing plant	September 2018	Sogn og Fjordane
MF7056	rural/urban	yes	ST-1	CC1	I	PRJNA689486	SAMN17211720	JAEPJD000000000	SRR13590740	406	Near food processing plant	September 2018	Sogn og Fjordane
MF7057	rural/urban	yes	ST-91	CC91	II	PRJNA689486	SAMN17211721	JAEOZG000000000	SRR13590739	426	Agricultural field	September 2018	Troms
MF7058	rural/urban	yes	ST-91	CC91	II	PRJNA689486	SAMN17211722	JAEPJC000000000	SRR13590738	427	Agricultural field	September 2018	Troms
MF7059	rural/urban	yes	ST-91	CC91	II	PRJNA689486	SAMN17211723	JAEPJB000000000	SRR13590737	429	Beach or sandbank	September 2018	Troms
MF7060	rural/urban	yes	ST-394	CC415	II	PRJNA689486	SAMN17211724	JAEPJA000000000	SRR13590736	425	Agricultural field	September 2018	Troms
MF7061	rural/urban	yes	ST-451	CC11	II	PRJNA689486	SAMN17211725	JAEPIZ000000000	SRR13590735	98	Grazeland or animal path	October 2018	Akershus
MF7062	rural/urban	yes	ST-451	CC11	II	PRJNA689486	SAMN17211726	JAEPIY000000000	SRR13590734	121	Urban or residential area	October 2018	Akershus
MF7063	rural/urban	yes	ST-398	CC19	II	PRJNA689486	SAMN17211727	JAEPIX000000000	SRR13590733	123	Urban or residential area	October 2018	Akershus
MF7603	rural/urban	yes	ST-399	CC14	II	PRJNA689486	SAMN17211728	JAEOZF000000000	SRR13590732	53	Grazeland or animal path	September 2019	Akershus

MF7604	rural/urban	yes	ST-399	CC14	II	PRJNA689486	SAMN17211729	JAEOZE000000000	SRR13590730	54	Grazeland or animal path	September 2019	Akershus
MF7605	rural/urban	yes	ST-399	CC14	II	PRJNA689486	SAMN17211730	JAEOZD000000000	SRR13590729	55	Grazeland or animal path	September 2019	Akershus
MF7606	rural/urban	yes	ST-398	CC19	II	PRJNA689486	SAMN17211731	JAEOZC000000000	SRR13590728	56	Grazeland or animal path	September 2019	Akershus
MF7607	rural/urban	yes	ST-451	CC11	II	PRJNA689486	SAMN17211732	JAEOZB000000000	SRR13590727	48	Urban or residential area	September 2019	Akershus
MF7608	rural/urban	yes	ST-425	CC90	II	PRJNA689486	SAMN17211733	JAEOZA000000000	SRR13590726	49	Urban or residential area	September 2019	Akershus
MF7609	rural/urban	yes	ST-4	CC4	I	PRJNA689486	SAMN17211734	JAEOYZ000000000	SRR13590725	66	Urban or residential area	September 2019	Akershus
MF7610	rural/urban	yes	ST-2343	CC37	II	PRJNA689486	SAMN17211735	JAEOYY000000000	SRR13590724	98	Grazeland or animal path	September 2019	Akershus
MF7611	rural/urban	yes	ST-37	CC37	II	PRJNA689486	SAMN17211736	JAEOYX000000000	SRR13590723	100	Grazeland or animal path	September 2019	Akershus
MF7612	rural/urban	yes	ST-91	CC91	II	PRJNA689486	SAMN17211737	JAEOYW000000000	SRR13590722	101	Grazeland or animal path	September 2019	Akershus
MF7613	rural/urban	yes	ST-91	CC91	II	PRJNA689486	SAMN17211738	JAEOYV000000000	SRR13590721	106	Grazeland or animal path	September 2019	Akershus
MF7614	rural/urban	yes	ST-1813	CC671	II	PRJNA689486	SAMN17211739	JAEOYU000000000	SRR13590719	120	Urban or residential area	September 2019	Akershus
MF7615	rural/urban	yes	ST-398	CC19	II	PRJNA689486	SAMN17211740	JAEOYT000000000	SRR13590718	121	Urban or residential area	September 2019	Akershus
MF7616	rural/urban	yes	ST-91	CC91	II	PRJNA689486	SAMN17211741	JAEOYS000000000	SRR13590717	129	Urban or residential area	September 2019	Akershus
MF7617	rural/urban	yes	ST-121	CC121	II	PRJNA689486	SAMN17211742	JAEOYR000000000	SRR13590716	130	Urban or residential area	September 2019	Akershus
MF7618	rural/urban	yes	ST-20	CC20	II	PRJNA689486	SAMN17211743	JAEOYQ000000000	SRR13590715	133	Urban or residential area	September 2019	Akershus
MF7619	rural/urban	yes	ST-4	CC4	I	PRJNA689486	SAMN17211744	JAEOYP000000000	SRR13590714	134	Urban or residential area	September 2019	Akershus
MF7620	rural/urban	yes	ST-37	CC37	II	PRJNA689486	SAMN17211745	JAEOYO000000000	SRR13590713	251	Urban or residential area	September 2019	Oslo
MF7621	rural/urban	yes	ST-398	CC19	II	PRJNA689486	SAMN17211746	JAEOYN000000000	SRR13590712	252	Grazeland or animal path	September 2019	Oslo
MF7622	rural/urban	yes	ST-204	CC204	II	PRJNA689486	SAMN17211747	JAEOYM000000000	SRR13590711	259	Urban or residential area	September 2019	Oslo
MF7623	rural/urban	yes	ST-204	CC204	II	PRJNA689486	SAMN17211748	JAEOYL000000000	SRR13590710	262	Urban or residential area	September 2019	Oslo
MF7624	rural/urban	yes	ST-451	CC11	II	PRJNA689486	SAMN17211749	JAEOYK000000000	SRR13590708	268	Urban or residential area	September 2019	Oslo
MF7625	rural/urban	yes	ST-4	CC4	I	PRJNA689486	SAMN17211750	JAEOYJ000000000	SRR13590707	33	Urban or residential area	September 2019	Akershus
MF7790	rural/urban	yes	ST-451	CC11	II	PRJNA689486	SAMN17211751	JAEOYI000000000	SRR13590706	54	Grazeland or animal path	January 2020	Akershus
MF7791	rural/urban	yes	ST-91	CC91	II	PRJNA689486	SAMN17211752	JAEOYH000000000	SRR13590705	55	Grazeland or animal path	January 2020	Akershus
MF7792	rural/urban	yes	ST-6	CC6	I	PRJNA689486	SAMN17211753	JAEOYG000000000	SRR13590704	56	Grazeland or animal path	January 2020	Akershus
MF7793	rural/urban	yes	ST-91	CC91	II	PRJNA689486	SAMN17211754	JAEOYF000000000	SRR13590703	98	Grazeland or animal path	January 2020	Akershus
MF7794	rural/urban	yes	ST-91	CC91	II	PRJNA689486	SAMN17211755	JAEOYE000000000	SRR13590702	99	Grazeland or animal path	January 2020	Akershus
MF7795	rural/urban	yes	ST-398	CC19	II	PRJNA689486	SAMN17211756	JAEOYD000000000	SRR13590701	120	Urban or residential area	January 2020	Akershus
MF7796	rural/urban	yes	ST-398	CC19	II	PRJNA689486	SAMN17211757	JAEOYC000000000	SRR13590700	121	Urban or residential area	January 2020	Akershus
MF7797	rural/urban	yes	ST-20	CC20	II	PRJNA689486	SAMN17211758	JAEOYB000000000	SRR13590699	134	Urban or residential area	January 2020	Akershus
MF7798	rural/urban	yes	ST-37	CC37	II	PRJNA689486	SAMN17211759	JAEOYA000000000	SRR13590812	53	Grazeland or animal path	January 2020	Akershus
MF7800	rural/urban	yes	ST-37	CC37	II	PRJNA689486	SAMN17211760	JAEOXZ000000000	SRR13590811	251	Urban or residential area	January 2020	Oslo
MF7801	rural/urban	yes	ST-7	CC7	II	PRJNA689486	SAMN17211761	JAEOXY000000000	SRR13590810	252	Grazeland or animal path	January 2020	Oslo
MF7802	rural/urban	yes	ST-425	CC90	II	PRJNA689486	SAMN17211762	JAEOXX000000000	SRR13590809	253	Urban or residential area	January 2020	Oslo
MF7803	rural/urban	yes	ST-398	CC19	II	PRJNA689486	SAMN17211763	JAEOXW000000000	SRR13590808	259	Urban or residential area	January 2020	Oslo
MF7804	rural/urban	yes	ST-120	CC8	II	PRJNA689486	SAMN17211764	JAEOXV000000000	SRR13590807	262	Urban or residential area	January 2020	Oslo
MF7805	rural/urban	yes	ST-425	CC90	II	PRJNA689486	SAMN17211765	JAEOXU000000000	SRR13590806	48	Urban or residential area	January 2020	Akershus
MF7806	rural/urban	yes	ST-425	CC90	II	PRJNA689486	SAMN17211766	JAEOXT000000000	SRR13590805	49	Urban or residential area	January 2020	Akershus
MF7831	rural/urban	yes	ST-647	CC20	II	PRJNA689486	SAMN17211767	JAEOXS000000000	SRR13590804	406	Near food processing plant	January 2020	Sogn og Fjordane
MF7832	rural/urban	yes	ST-647	CC20	II	PRJNA689486	SAMN17211768	JAEOXR000000000	SRR13590803	363	Near food processing plant	January 2020	Sogn og Fjordane
MF7833	rural/urban	yes	ST-7	CC7	II	PRJNA689486	SAMN17211769	JAEOXQ000000000	SRR13590801	595	Near food processing plant	February 2020	Østfold
MF7834	rural/urban	yes	ST-220	CC220	I	PRJNA689486	SAMN17211770	JAEOXP000000000	SRR13590800	598	Near food processing plant	February 2020	Østfold
MF8003	rural/urban	yes	ST-398	CC19	II	PRJNA689486	SAMN17211771	JAEOXO000000000	SRR13590799	609	Agricultural field	April 2020	Vestfold
MF8004	rural/urban	yes	ST-8	CC8	II	PRJNA689486	SAMN17211772	JAEOXN000000000	SRR13590798	656	Agricultural field	April 2020	Agder
MF8008	rural/urban	yes	ST-7	CC7	II	PRJNA689486	SAMN17211773	JAEOXM000000000	SRR13590797	730	Forest or mountain area	April 2020	Rogaland
MF8009	rural/urban	yes	ST-37	CC37	II	PRJNA689486	SAMN17211774	JAEOXL000000000	SRR13590796	732	Agricultural field	April 2020	Rogaland
MF8010	rural/urban	yes	ST-37	CC37	II	PRJNA689486	SAMN17211775	JAEPIW000000000	SRR13590795	734	Agricultural field	April 2020	Rogaland
MF8015	slugs	no	ST-220	CC220	I	-	-	-	-	NMBU 4305, Hygiberia 1		2012	Hordaland
MF8016	slugs	yes	ST-8	CC8	II	PRJNA689487	SAMN17210413	JAENVR000000000	SRR13360783	NMBU 4306, Hygiberia 2		2012	Aust Agder
MF8017	slugs	no	ST-759	CC20	II	-	-	-	-	NMBU 4307, Hygiberia 3		2012	Buskerud
MF8018	slugs	yes	ST-14	CC14	II	PRJNA689487	SAMN17210414	JAENVQ000000000	SRR13360782	NMBU 4308, Hygiberia 4		2012	Hordaland
MF8019	slugs	yes	ST-398	CC19	II	PRJNA689487	SAMN17210415	JAENVP000000000	SRR13360771	NMBU 4309, Hygiberia 5		2012	Akershus
MF8020	slugs	yes	ST-7	CC7	II	PRJNA689487	SAMN17210416	JAENVO000000000	SRR13360766	NMBU 4310, Hygiberia 6		2012	Buskerud
MF8021	slugs	yes	ST-6	CC6	I	PRJNA689487	SAMN17210417	JAENVN000000000	SRR13360765	NMBU 4311, Hygiberia 7		2012	Hordaland
MF8022	slugs	yes	ST-37	CC37	II	PRJNA689487	SAMN17210418	JAENVM000000000	SRR13360764	NMBU 4312, Hygiberia 8		2012	Sør-Trøndelag
MF8023	slugs	yes	ST-1	CC1	I	PRJNA689487	SAMN17210419	JAENVL000000000	SRR13360763	NMBU 4313, Hygiberia 9		2012	Akershus
MF8024	slugs	yes	ST-6	CC6	I	PRJNA689487	SAMN17210420	JAENVK000000000	SRR13360762	NMBU 4314, Hygiberia 10		2012	Hordaland

MF8025	slugs	yes	ST-7	CC7	II	PRJNA689487	SAMN17210421	JAENVJ000000000	SRR13360761	NMBU 4315, Hygiberia 11		2012	Oslo
MF8026	slugs	yes	ST-18	CC18	II	PRJNA689487	SAMN17210422	JAENVI000000000	SRR13360760	NMBU 4316, Hygiberia 12		2012	Telemark
MF8027	slugs	no	ST-124	CC124	II	-	-	-	-	NMBU 4317, Hygiberia 13		2012	Telemark
MF8028	slugs	yes	ST-7	CC7	II	PRJNA689487	SAMN17210423	JAENVH000000000	SRR13360781	NMBU 4318, Hygiberia 14		2012	Sør-Trøndelag
MF8029	slugs	no	ST-1	CC1	I	-	-	-	-	NMBU 4319, Hygiberia 15		2012	Møre og Romsdal
MF8030	slugs	no	ST-91	CC91	II	-	-	-	-	NMBU 4320, Hygiberia 16		2012	Møre og Romsdal
MF8031	slugs	no	ST-399	CC14	II	-	-	-	-	NMBU 4321, Hygiberia 17		2012	Vestfold
MF8032	slugs	yes	ST-4	CC4	I	PRJNA689487	SAMN17210424	JAENVG000000000	SRR13360780	NMBU 4322, Hygiberia 18		2012	Akershus
MF8033	slugs	yes	ST-1	CC1	I	PRJNA689487	SAMN17210425	JAENVF000000000	SRR13360779	NMBU 4323, Hygiberia 19		2012	Sogn og Fjordene
MF8034	slugs	yes	ST-1	CC1	I	PRJNA689487	SAMN17210426	JAENVE000000000	SRR13360778	NMBU 4324, Hygiberia 20		2012	Akershus
MF8035	slugs	yes	ST-19	CC19	II	PRJNA689487	SAMN17210427	JAENVD000000000	SRR13360777	NMBU 4325, Hygiberia 21		2012	Hordaland
MF8036	slugs	yes	ST-14	CC14	II	PRJNA689487	SAMN17210428	JAENVC000000000	SRR13360776	NMBU 4326, Hygiberia 22		2012	Akershus
MF8037	slugs	no	ST-219	CC4	I	-	-	-	-	NMBU 4327, Hygiberia 23		2012	Rogaland
MF8039	slugs	yes	ST-91	CC91	II	PRJNA689487	SAMN17210429	JAENVB000000000	SRR13360775	NMBU 4329, Hygiberia 25		2012	Rogaland
MF8040	slugs	no	ST-120	CC8	II	-	-	-	-	NMBU 4330, Hygiberia 26		2012	Østfold
MF8041	slugs	no	ST-8	CC8	II	-	-	-	-	NMBU 4331, Hygiberia 27		2012	Sør-Trøndelag
MF8042	slugs	yes	ST-91	CC91	II	PRJNA689487	SAMN17210430	JAENVA000000000	SRR13360774	NMBU 4332, Hygiberia 28		2012	Møre og Romsdal
MF8043	slugs	yes	ST-18	CC18	II	PRJNA689487	SAMN17210431	JAENUZ000000000	SRR13360773	NMBU 4333, Hygiberia 29		2012	Vestfold
MF8044	slugs	yes	ST-1	CC1	I	PRJNA689487	SAMN17210432	JAENUY000000000	SRR13360772	NMBU 4334, Hygiberia 30		2012	Hordaland
MF8046	slugs	yes	ST-8	CC8	II	PRJNA689487	SAMN17210433	JAENUX000000000	SRR13360770	NMBU 4336, Hygiberia 32		2012	Vestfold
MF8047	slugs	yes	ST-91	CC91	II	PRJNA689487	SAMN17210434	JAENUW000000000	SRR13360769	NMBU 4337, Hygiberia 33		2012	Østfold
MF8048	slugs	no	ST-217	CC217	I	-	-	-	-	NMBU 4338, Hygiberia 34		2012	Rogaland
MF8049	slugs	yes	ST-9	CC9	II	PRJNA689487	SAMN17210435	JAENUV000000000	SRR13360768	NMBU 4339, Hygiberia 35		2012	Rogaland
MF8050	slugs	yes	ST-403	CC403	II	PRJNA689487	SAMN17210436	JAENUU000000000	SRR13360767	NMBU 4340, Hygiberia 36		2012	Rogaland

Supplemental S2 Table: Clusters of isolates from rural or urban environments

Cluster no.	Clonal Complex (ST)	No. of isolates in cluster*	No. of allelic differences between individual isolates or clusters*		Sampling site description	Year(s) collected	Sampling site no.	Max. distance between sampling sites	Geographic location	Isolates involved*
			wgMLST	cgMLST						
Clusters of isolates found at same site or nearby location on multiple sampling occasions (persistent clones)										
1	CC1 (ST1)	2	4	0	Quay outside fish processing plant	2017, 2018	287, 406	18 m	Sogn og Fjordane	MF6840, MF7056
2	CC4 (ST4)	3	13-14	0-1	Soil from grazeland for cattle; decaying vegetation by feeding station; and decaying vegetation at edge of pond	2016, 2017, 2019	53, 109, 134	1.3 km	Ås, Akershus	MF6716, MF6554, MF7619
3	CC7 (ST732)	8	2-23	0	Quay outside fish processing plant	2017, 2018	279, 390, 391, 394, 363, 375	75 m	Sogn og Fjordane	MF6838, MF6988, MF7046, MF7051, MF7052, MF7053, MF7054, MF7055
4	CC11 (ST451)	4	1-10	0	Garden compost heap	2016, 2019	48, 49, 50	3 m	Ås, Akershus	MF6550, MF7607, MF6551, MF6552
5	CC11 (ST451)	2	15	0	Agricultural area and next to park bench in town centre	2018, 2020	54, 121	2.3 km	Ås, Akershus	MF7062, MF7790
6	CC91 (ST91)	3	4, 8, and 34	0	Agricultural area, sheep grazing pasture	2016, 2017	54, 56, 113	1 km	Ås, Akershus	MF6555, MF6557, MF6717
7a	CC91 (ST91)	4	2-5	0	Agricultural area, sheep grazing pasture	2019, 2020	98, 99, 101, 106	500 m	Ås, Akershus	MF7612, MF7613, MF7793, MF7794
7b		(4)+1	11-12	0	Agricultural area, sheep grazeland and flowerbed in town centre	2017, 2019, 2020	(98, 99, 101, 106) + 120	1.5 km	Ås, Akershus	(MF7612, MF7613, MF7793, MF7794) + MF6720
8	CC19 (ST398)	3	2	0	Flowerbed and next to adjacent park bench	2019, 2020	120, 121	1 m	Ås, Akershus	MF7795, MF7796, MF7615
9	CC20 (ST20)	2	0	0	Decaying vegetation at edge of pond	2019, 2020	133, 134	4 m	Ås, Akershus	MF7618, MF7797
10	CC20 (ST647)	4	2-3	0	Quay outside fish processing plant	2018, 2020	287, 354, 363, 406	75 m	Sogn og Fjordane	MF7048, MF7049, MF7831, MF7832
11	CC37 (ST37)	2	7	0	Decaying leaves/vegetation on bike path	2019, 2020	251	0 m	Oslo	MF7620, MF7800
12	CC37 (ST37)	2	3	0	Agricultural area, sheep grazing pasture	2017, 2019	53, 115	1 km	Ås, Akershus	MF6718, MF7798
13	CC90 (ST425)	3	0-2	0	Garden compost heap	2019, 2020	48, 49	1 m	Ås, Akershus	MF7805, MF7608, MF7806
14	CC204 (ST204)	3	3-10	0	Flowerbed with pigeons; soil by treeroot in city centre	2017, 2019	259, 262	300 m	Oslo	MF6830, MF7622, MF7623
Cluster of isolates found at more distant locations (>3 km)										
15	CC6 (ST6)	2	9	0	Sheep grazing pasture; soil by treeroot in city centre	2017, 2020	56, 262	30 km	Ås, Akershus and Oslo	MF7792, MF6831
Clusters of isolates found at the same location at the same time										
16	CC4 (ST4)	2	4	0	Puddle next to road and brook in residential area	2019	33, 66	1.1 km	Akershus	MF7609, MF7625
17	CC91 (ST91)	3	7-10	0-1	Agricultural field and nearby beach	2018	426, 427, 429	150 m	Troms	MF7057, MF7058, MF7059
18	CC91 (ST91)	2	8	0	Soil samples close to edge of pond	2017	130, 132	30 m	Ås, Akershus	MF6724, MF6726
19	CC18 (ST18)	3	1-2	0	Soil from or near grazeland for cattle;	2017	184, 185, 188	73 m	Troms	MF6805, MF6806, MF6807
20	CC37 (ST37)	2	1	0	Agricultural field	2020	732, 734	35 m	Rogaland	MF8009, MF8010
21	CC19 (ST398)	7	1-6	0	Agricultural field and in nearby town centre	2017	98, 99, 100, 101, 118, 123, 133	1.5 km	Ås, Akershus	MF6711, MF6712, MF6713, MF6714, MF6719, MF6722, MF6727

**When the number of isolates is listed as two groups of isolates [i.e. (4)+1], the differences between the isolates enclosed by a parenthesis is not reported*

Supplemental S3 Table: Clusters of dairy farms isolates

Cluster no.	Clonal Complex (ST)	No. of isolates in cluster*	No. of allelic differences between individual isolates or clusters*		Sample type	Farm no.	Visit no.	Isolates involved*	Comments
			wgMLST	cgMLST					
Clusters of isolates found on the same farm on the same visit									
1	CC11 (ST451)	2	1	0	Feed and feces samples	Farm 12	Visit 3	D145L, D092L	also found on multiple visits
2	CC11 (ST451)	2	1	0	Feed and feces samples	Farm 6	Visit 4	D118L, D117L	also found on multiple visits
3	CC91 (ST91)	2	1	0	Milk filter and feces samples	Farm 12	Visit 5	D169L, D158L	also found on multiple visits
4	CC91 (ST91)	2	0	0	Milk filter and teat swab samples	Farm 13	Visit 5	D204L, D162L	
5	CC91 (ST91)	2	1	0	Feed and feces samples	Farm 14	Visit 4	D140L, D137L	
6	CC18 (ST18)	2	3	0	Feed and feces samples	Farm 1	Visit 2	D031Lb, D032L	also found on multiple visits
7	CC18 (ST18)	2	3	0	Milk filter and feces samples	Farm 1	Visit 3	D141L, D077L	also found on multiple visits
8	CC18 (ST18)	2	0	0	Feed and feces samples	Farm 17	Visit 3	D103L, D146L	
9	CC18 (ST2761)	2	2	0	Feed and feces samples	Farm 3	Visit 3	D081L, D143L	also found on multiple visits
10	CC20 (ST20)	2	1	0	Teat swab and feces samples	Farm 1	Visit 4	D116L, D142L	
11	CC177 (ST177)	3	1-2	0	Feed, feces, and milk filter samples	Farm 12	Visit 4	D136L, D138L, D139L	also found on multiple visits
Clusters of isolates found on the same farm on multiple visits (persistent clones)									
12	CC4 (ST4)	3	2-11	0	Two feces and one feed sample	Farm 11	Visits 1, 3, and 6	D010L, D087L, D182L	-
13	CC8 (ST8)	2	1	0	Feed and feces samples	Farm 13	Visits 2 and 5	D161L, D052L	-
14	CC11 (ST451)	3	1-7	0	Milk filter, feed, and feces	Farm 12	Visits 2 and 3	D145L, D092L, D066L	includes cluster 1
15	CC11 (ST451)	3	1-3	0	Two feed and one feces sample	Farm 6	Visits 2 and 4	D118L, D117L, D044L	includes cluster 2
16	CC91 (ST91)	3	1-3	0	Milk filter, feed, and feces samples	Farm 12	Visits 2 and 5	D169L, D158L, D051L	includes cluster 3
17	CC18 (ST2761)	3	2-2	0	Two feces and one feed sample	Farm 3	Visits 3 and 4	D081L, D143L, D108L	includes cluster 9
18	CC18 (ST18)	4	1-5	0	One milk filter, two feces, and one feed sample	Farm 1	Visits 2 and 3	D031Lb, D032L, D141L, D077L	comprises clusters 6 and 7
19	CC37 (ST37)	2	3	0	Teat swab and feed sample	Farm 12	Visits 1 and 5	D029L, D159L	-
20	CC177 (ST177)	4	1-2	0	One milk filter, one feces, and two feed samples	Farm 12	Visits 4 and 6	D136L, D138L, D139L, D192L	includes cluster 11
21	CC226 (ST226)	2	9	0	Two feed samples	Farm 5	Visits 1 and 2	D001L, D038L	-
22	CC412 (ST412)	2	6	0	Feed and feces samples	Farm 10	Visits 4 and 6	D189L, D135L	-
23	CC415 (ST394)	2	0	0	Two feed samples	Farm 2	Visits 3 and 4	D107L, D078L	-
Clusters of isolates found on more than one farm									
24	CC11 (ST451)	(3)+5	15-57	0-1	Three milk filters, two feed and three feces samples	Farms 4, 6, 9, 11, and 12	Visits 2, 4, and 6	(D118L, D117L, D044L), D201L, D050L, D191L, D034L, D130L	includes cluster 15
25	CC11 (ST451)	2	19	0	Milk filter and feed samples	Farms 8 and 9	Visit 6	D200L, D203L	-
26	CC226 (ST226)	(2)+1	9-12	0	Three feed samples	Farms 4 and 5	Visits 1 and 2	(D001L, D038L), D036L	includes cluster 21
27	CC415 (ST394)	(2)+1	11	1	Feed and feces samples	Farms 2 and 5	Visits 2, 3, and 4	(D107L, D078L), D037L	includes cluster 23

**When the number of isolates is listed as two groups of isolates [e.g. (3)+5], the differences between the isolates enclosed by a parenthesis is not reported*

Supplemental S4 Table: Clusters containing isolates from both rural/urban environments and dairy farms

Cluster no.	Clonal Complex (ST)	No. of isolates in cluster*	No. of allelic differences between individual isolates or clusters*		Farm sample(s)			Sample(s) from rural/urban environments		Isolates involved*
			wgMLST	cgMLST	Sample type	Farm no.	Visit (in 2019-2020)	Sampling site description	Year(s) collected	
1	CC6 (ST6)	3	9-18	0	Feed sample	Farm 17	Visit 1	Sheep grazing pasture in Ås and at foot of tree in Oslo city centre	2017, 2020	D011L, MF7792, MF6831
2	CC11 (ST451)	(8)+(4)	15-27	0-1	Three milk filters, two feed and three feces samples	Farms 4, 6, 9, 11, and 12	Visits 2, 4, and 6	Garden compost heap in Ås	2016, 2019	(D034L, D044L, D050L, D117L, D118L, D130L, D191L, D201L), (MF6550, MF7607, MF6551, MF6552)
3	CC91 (ST91)	1+(4)	20-21	1	Feed sample	Farm 18	Visit 5	Agricultural area, sheep grazing pasture in Ås	2019, 2020	D170L, (MF7612, MF7613, MF7793, MF7794)
4	CC37 (ST37)	(2)+(2)	9-14	0	Teat swab and feed sample	Farm 12	Visits 1 and 5	Two isolates from agricultural area and sheep grazing pasture in Ås isolated ~1 km apart	2017, 2020	(D029L, D159L), (MF6718, MF7798)
5	CC37 (ST37)	(2)+1	15-16	0	Feed sample	Farm 1	Visit 4	Two isolates from grazing land/pasture in Rogaland	2020	D115L, (MF8009, MF8010)
6	CC415 (ST394)	(3)+1	21-23	0-1	Two feed and one feces sample	Farms 2 and 5	Visits 2, 3, and 4	Agricultural field in Troms	2018	(D107L, D078L, D037L), MF7060

**When the number of isolates is listed as two groups of isolates [e.g. (2)+(2)], the differences between the isolates enclosed by a parenthesis is not reported*

Supplemental S5 Table: Clusters containing isolates from slugs or both slugs and rural/urban/farm environments

Clusters containing slug isolates

Cluster no.	Clonal Complex (ST)	No. of isolates in cluster	wgMLST differences between individual isolates		Geographic locations	Isolates involved
			wgMLST	cgMLST		
1	CC1 (ST1)	2	11	0	Two different locations in Akershus	MF8023, MF8034
2	CC14 (ST14)	2	2	0	Hordaland and Akershus	MF8018, MF8036

Clusters containg isolates from both slugs and either rural/urban or dairy farm environments

Cluster no.	Clonal Complex (ST)	No. of isolates in cluster*	wgMLST differences between individual isolates or clusters*		Geographic location slug isolate	Sample from rural/urban/farm environments		
			wgMLST	cgMLST		Sampling point description	Year(s) collected	Isolates involved*
3	CC1 (ST1)	2	10	0	Hordaland	Roadside curb in residential area in Oslo	2017	MF8044, MF6829
4	CC7 (ST7)	2	15	2	Sør-Trøndelag	Soil near horse paddock in Oslo	2020	MF8028, MF7801
5	CC19 (ST398)	1+(3)	17-19	0	Akershus	Flowerbed and next to adjacent park bench in Ås	2019, 2020	MF8019, (MF7795, MF7796, MF7615)
6	CC4 (ST4)	2	21	3	Akershus	Soil from grazeland for cattle in Ås	2017	MF8032, MF6716
7	CC6 (ST6)	2	21	0	Hordaland	Feed sample from Farm 17	2019	MF8024, D011L

**When the number of isolates is listed as two groups of isolates [i.e. 1+(3)], the differences between the isolates enclosed by a parenthesis is not reported*

Supplemental S6 Table: Clusters of clinical isolates

Cluster no.	Clonal Complex (ST)	No. of isolates in cluster	No. of allelic differences between isolates		Year(s) collected	Isolates
			wgMLST	cgMLST		
Pairs of clinical isolates with ≤ 10 cgMLST differences (not belonging to larger clusters)						
1	CC7 (ST7)	2	6	1	2011, 2012	ERR2522266, ERR2522288
2	CC7 (ST7)	2	9	1	2012, 2014	ERR2522305, ERR2522345
3	CC7 (ST7)	2	13	0	2014, 2015	ERR2522334, ERR2522359
4	CC8 (ST8)	2	5	0	2013, 2015	ERR2522319, ERR2522362
5	CC11 (ST451)	2	2	0	2013	ERR2522314, ERR2522323
6	CC14 (ST14)	2	5	0	2011, 2012	ERR2522279, ERR2522283
7	CC18 (ST18)	2	5	0	2015	ERR2522356, ERR2522357
8	CC18 (ST18)	2	73	8	2010, 2014	ERR2522259, ERR2522332
9	CC20 (ST20)	2	59	8	2012	ERR2522296, ERR2522307
10	CC88 (ST296)	2	7	0	2015	ERR2522354, ERR2522358
11	CC101 (ST101)	2	5	0	2010	ERR2522249, ERR2522255
12	CC121 (ST121)	2	10	6	2010, 2014	ERR2522258, ERR2522335
13	CC177 (ST177)	2	73	2	2012, 2014	ERR2522308, ERR2522327
14	CC226 (ST226)	2	53	8	2014, 2015	ERR2522349, ERR2522361
15	CC415 (ST394)	2	24	10	2011	ERR2522269, ERR2522265
Clusters of >2 clinical isolates with ≤10 cgMLST differences						
16	CC7 (ST7)	6	1-15	0-3	2014	ERR2522328, ERR2522329, ERR2522331, ERR2522343, ERR2522347, ERR2522351
17	CC7 (ST7)	3	10-17	0-2	2×2010, 2015	ERR2522247, ERR2522257, ERR2522365
18	CC8 (ST8)	4	12-57	0-4	2010, 2012, 2013, 2014	ERR2522243, ERR2522286, ERR2522325, ERR2522346
19*	CC19 (ST802)	4	0-2	0	2013	ERR2522318, ERR2522320, ERR2522321, ERR2522324
20	CC37 (ST37)	3	24-37	8-9	2013, 2015, 2018	ERR2522315, ERR2522368, ERR3446056
21	CC87 (ST87)	5	3-11	0-2	2010, 2011, 2012, 2×2015	ERR2522244, ERR2522281, ERR2522300, ERR2522364, ERR2522366
22	CC121 (ST121)	3	5-24	1-8	2011, 2×2014	ERR2522280, ERR2522340, ERR2522352
23	CC7 (ST7)	8	1-105	0-10	2010, 2011, 5×2012, 2015	ERR2522253, ERR2522282, ERR2522299, ERR2522302, ERR2522303, ERR2522277, ERR2522287, ERR2522360
Included within cluster 23		5	1-6	0-1	2010, 4×2012	ERR2522253, ERR2522282, ERR2522299, ERR2522302, ERR2522303
24	CC121 (ST121)	10	2-55	0-12	5×2012, 2013, 2×2014, 2×2015	ERR2522292, ERR2522338, ERR2522312, ERR2522295, ERR2522337, ERR2522294, ERR2522297, ERR2522284, ERR2522353, ERR2522363
Included within cluster 24		3	12-19	3-8	2012, 2013, 2014	ERR2522292, ERR2522338, ERR2522312
Included within cluster 24		3	2-5	0	2×2012, 2014	ERR2522295, ERR2522337, ERR2522294
Included within cluster 24		7	2-20	0-8	4×2012, 2014, 2×2015	ERR2522295, ERR2522337, ERR2522294, ERR2522297, ERR2522284, ERR2522353, ERR2522363

** Known outbreak associated with fermented fish: https://www.fhi.no/sv/utbrudd/oversikt-over-storre-utbrudd/utbrudd-av-listeriose-i-norge/*

Supplemental S7 Table: Clusters containing both clinical isolates and isolates from rural/urban/farm environments or slugs

Cluster no.	Clonal Complex (ST)	No. of isolates in cluster*	No. of allelic differences between individual isolates or clusters*		Clinical isolate(s)		Environmental isolates		
			wgMLST	cgMLST	Isolate(s)	Year(s) collected	Isolate(s)	Sampling site description	Year(s) collected
1	CC4 (ST4)	1+3	20-23	5-6	ERR2522342	2014	MF6716, MF6554, MF7619	Grazeland for cattle and decaying vegetation at edge of pond, in Ås (1.3 km between samling sites)	2016, 2017, 2019
2a	CC7 (ST7)	3+1	9-18	1-3	ERR2522257, ERR2522365, ERR2522247	2010, 2015	MF8028	Slug isolate from Sør-Trøndelag	2012
2b		3+1	12-21	1-3			MF7801	Soil near horse paddock in Oslo	2020
3	CC7 (ST7)	5+1	11-15	0-1	ERR2522299, ERR2522253, ERR2522282, ERR2522302, ERR2522303	2010, 2012	MF7833	Near food processing plant	2020
4	CC8 (ST8)	2+1	14-15	0-1	ERR2522286, ERR2522325	2012, 2013	MF8046	Slug isolate from Vestfold	2012
5	CC9 (ST9)	1+1	6	0	ERR2522369	2015	MF8049	Slug isolate from Rogaland	2012
6a	CC11 (ST451)	2+1	20-21	2	ERR2522314, ERR2522323	2013	MF7062	Next to park bench in town centre in Ås	2018
6b		2+1	9-11	1			D050L	Dairy farm isolate; milk filter from farm 11	2019
6c		2+7	14-23	1-3			D034L, D044L, D117L, D118L, D130L, D191L, D201L	Dairy farm isolates	2019, 2020
6d		2+1	15-17	1			MF7624	Puddle on gravel path, city park	2019
6e		2+4	16-21	2			MF6550, MF6551, MF6552, MF7607	Garden compost heap	2016, 2019
7	CC220 (ST220)	1+1	19	5	ERR2522322	2013	MF7834	Roadside close to storm drain in Østfold	2020
8	CC403 (ST403)	1+1	22	7	ERR2522348	2014	MF6834	Bird droppings from a park in Oslo	2017
9	CC415 (ST394)	2+1	19-21	3-7	ERR2522265, ERR2522269	2011	MF7060	Agricultural field in Troms	2018

**The number of isolates is listed as two groups of isolates (e.g. 1+3), representing clinical and environmental isolates, respectively. The number of wgMLST differences is given as the distance separating the isolates belonging to each group*

Supplementary material Paper II

Additional supplementary material related to this article can be found in the online version, available at https://doi.org/10.1128/aem.02136-21.

Paper III

foods

MDPI

Article

The Ability of Shiga Toxin-Producing *Escherichia coli* to Grow in Raw Cow's Milk Stored at Low Temperatures

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

[1] Department of Paraclinical Sciences, Faculty of Veterinary Medicine, Norwegian University of Life Sciences, 1432 Ås, Norway
[2] Department of Production Animal Clinical Sciences, Faculty of Veterinary Medicine, Norwegian University of Life Sciences, 1432 Ås, Norway
* Correspondence: toril.lindback@nmbu.no; Tel.: +47-97982726

Abstract: Despite the lack of scientific evidence, some consumers assert that raw milk is a natural food with nutritional and immunological properties superior to pasteurized milk. This has led to the increased popularity of unpasteurized cow milk (UPM) and disregard for the risks of being exposed to zoonotic infections. Dairy cattle are healthy carriers of Shiga toxin (Stx)-producing *E. coli* (STEC), and contaminated UPM has caused STEC outbreaks worldwide. The association between STEC, carrying the *eae* (*E. coli* attachment effacement) gene, and severe diseases is well-established. We have previously isolated four *eae* positive STEC isolates from two neighboring dairy farms in the Southeast of Norway. A whole genome analysis revealed that isolates from different farms exhibited nearly identical genetic profiles. To explore the risks associated with drinking UPM, we examined the ability of the isolates to produce Stx and their growth in UPM at different temperatures. All the isolates produced Stx and one of the isolates was able to propagate in UPM at 8 °C ($p < 0.02$). Altogether, these results highlight the risk for STEC infections associated with the consumption of UPM.

Keywords: Shiga toxin-producing *Escherichia coli*; raw cow's milk; unpasteurized; storage; temperature; food safety; Shiga-toxin; bacteriophage

Citation: Idland, L.; Bø-Granquist, E.G.; Aspholm, M.; Lindbäck, T. The Ability of Shiga Toxin-Producing *Escherichia coli* to Grow in Raw Cow's Milk Stored at Low Temperatures. *Foods* **2022**, *11*, 3411. https://doi.org/10.3390/foods11213411

Academic Editors: Piero Franceschi and Paolo Formaggioni

Received: 5 October 2022
Accepted: 22 October 2022
Published: 28 October 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

1. Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) is a globally distributed intestinal pathogen associated with human diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (HUS) [1]. The term "EHEC" is restricted to Shiga toxin-producing *E. coli* (STEC) associated with human disease. The main reservoir of STEC is the ruminant digestive tract and undercooked beef and unpasteurized milk are considered high-risk foods for STEC infections [1,2]. In 2020, 4446 cases of EHEC disease and 13 deaths were reported in the EU [3]. The first large outbreak of EHEC occurred in the USA in 1982 and was caused by a strain of serotype O157:H7 [4]. Since then, other serotypes have also been associated with outbreaks of EHEC disease [5–7]. The most known non-O157:H7 strain is O104:H4, which caused 855 cases of HUS and 50 fatalities during a large European outbreak in 2011 [2]. EHEC has a low infectious dose of 10–100 colony-forming units [8,9], and insufficient food decontamination practices increases the risk for EHEC infections.

STEC can produce two different types of Shiga toxin, Stx1 and Stx2, both comprising several subtypes. Stx2 is more often associated with HUS than Stx1, and Stx2a is considered as the most potent subtype of the toxin [2]. The Stx-encoding genes are carried by temperate bacteriophages [2], and the pathogenic potential of STEC has been suggested to be influenced by the "EHEC phage replication unit" (Eru) located in the phage genome [10,11]. The life cycle of temperate phages is regulated by the CI repressor protein, which represses the transcription of the replication proteins during the lysogenic state of the phage [12,13]. The de-repression of CI results in the production of Stx and new phage particles [14]. Based on similarities in its amino acid sequence, the CI protein of Stx phages has been grouped into

eight major clades (I–VIII) [11]. Exactly how the variability in the CI sequence influences its regulatory properties and potentially the virulence properties of its host STEC strain have not been explored so far.

Stx production combined with the ability to adhere to the intestinal epithelium via the adhesion protein intimin are believed to be necessary for STEC to cause severe disease. The intimin-encoding gene *(eae)* is part of the locus of the enterocyte effacement pathogenicity island (LEE-PAI), which encodes proteins responsible for introducing attaching and effacing (A/E) lesions to the epithelial cells [15]. Similar to CI, intimins display a structural diversity that potentially reflects differences in host cell tropism. The most common types of intimin are α, β, γ, ε, ζ, and η [15]. The β-type has been shown to predominate in non-O157 STEC strains from diarrheal patients, while cattle isolates more often carry the ζ-type [16]. The presence of *eae* is associated with a higher risk of developing HUS [17].

EHEC is regarded as an emerging public health challenge as new pathotypes and serotypes constantly appear [18–20]. Milk contaminated with pathogens causes foodborne disease worldwide, and 33% of all reported milk-borne disease outbreaks in England and Wales between 1992–2000 were caused by EHEC [21]. Previous studies have shown that 27, 13, and 5% of cattle from Portugal [22], US [23], and the EU [3] carry STEC, respectively. A study from Finland showed that 2% of on-farm, in-line milk filters were positive for STEC of the serotype O157:H7 [24], while in Norway, STEC has been detected in 7% of milk filters [25]. As STECs are carried by asymptomatic cows and frequently occur in dairy farm environments [26], the milk from these sources can easily be contaminated during the milking process. The lack of effective preventive measures in the primary production of milk makes pasteurization necessary to ensure food safety. Pasteurization at 72 °C for 15 s has shown to be very effective for the inactivation of STEC [27].

Low-temperature storage is important for preventing microbial growth in milk [28]. Previous studies have shown that STEC is not able to grow at 4 °C, but proliferation has been observed at inadequate refrigeration temperatures [29,30]. It has been shown that *E. coli* of the serotype O157:H7 grows in unpasteurized and pasteurized milk with a 2- to 3-log CFU/mL increase at 8 °C within a time period of seven days [31]. The European Food Safety Authority (EFSA) recommend that certain unpasteurized and low-pasteurized dairy products should be stored below 5 °C to minimize microbial growth [32]. However, the temperature in domestic refrigerators has been shown to vary between 7.0 ± 2.7 °C and 6.1 ± 2.8 °C for southern and northern European countries, respectively [33]. In addition, short breaks in the cold chain, for example, during meals, represent an additional but unexplored factor that may add to the risk of consuming UPM.

To further assess the food safety risk associated with the consumption of UPM, we need to gain more knowledge on the genetic- and growth characteristics of the STECs isolated from raw cows' milk. In the present study, we have compared the genome of four STECs isolated from milk [25] with a focus on their content of virulence-associated genes and Stx phages. The isolates were tested for their survival and growth in UPM milk, incubated at recommended and abused storage temperatures, and for the production of Stx at the body temperature of a human host. Altogether, the results highlight the risk for EHEC infections associated with the consumption of UPM, particularly if the milk has been stored at an abused temperature.

2. Materials and Methods

2.1. Culturing Conditions

This study comprises four *stx*- and *eae*-positive *E. coli* isolates from Norwegian dairy farms [25]. Three of the isolates were from the same farm, two from fecal samples (S2 and S4) and one from an in-line milk filter sample (S3), while the fourth isolate was isolated from a fecal sample (S1) at a nearby farm. The isolates were collected at two different sampling occasions separated by five months (Table 1). Raw milk, from the dairy cattle breed Norwegian Red, was collected from a bulk tank at the Center for Livestock Experiments at the Norwegian University of Life Sciences and used as cultivation medium in the growth

experiments. The milk was collected in batches of approximately 2 L at two different occasions (September 2021 and April 2022) and aliquoted in 40 mL batches in Falcon tubes and frozen at −20 °C until use.

Table 1. Characteristics of the Shiga toxin-producing *E. coli* isolated from dairy farms located in the southeast of Norway [25].

	S1	S2	S3	S4
Source	Cattle feces (Farm B)	Cattle feces (Farm A)	Milk filter (Farm A)	Cattle feces (Farm A)
Year of isolation	2019 (November)	2020 (January)	2020 (June)	2020 (June)
Country	Norway	Norway	Norway	Norway
Pathotype	STEC	STEC	STEC	STEC
Serotype	ONT:H28	O108:H25	ONT:H28	ONT:H28
NCBI accession no	JANWGF000000000	JANWGE000000000	JANWGD000000000	JANWGC000000000
LEE operons	five	five	five	five
Intimin type	gamma	alpha	gamma	gamma
ehxA	yes	yes	yes	yes
astA ST toxin	yes	yes (2)	yes	yes
Stx type	Stx1a	Stx2a	Stx1a	Stx1a
Eru type	lambdoid	Eru1	lambdoid	lambdoid
Stx phage CI clade	V	II	V	V

To explore the ability of the STEC isolates to grow at different temperatures, over-night cultures of the respective isolates grown in Lysogeny broth (LB) were diluted to OD_{600} = 0.3, whereof 0.5 µL were transferred to 40 mL of thawed raw milk. Immediately after inoculation, 10 µL of the milk samples was plated on CHROMagar™ STEC (Kanto Chemical Co., Tokyo, Japan) and incubated at 37 °C for 24 h to enumerate the start concentration of STEC. The inoculated raw milk samples were then incubated at five different temperature settings: optimal refrigerator temperature (4 °C), abused refrigerator temperatures (6 °C and 8 °C), room temperature (20 °C), and a temperature setting mimicking the situation when milk is kept at room temperature during meals (4 °C except for 1.5 h daily at 20 °C). To determine the temperature fluctuation of the samples incubated this way, the temperature was recorded in an uninoculated 40 mL raw milk sample every 15 min during the 20 °C incubation and until the milk temperature had returned to 4 °C, which encompassed a total time of 4.5 h. For enumeration of STEC in the raw milk samples incubated at different temperatures, dilutions of the samples were plated on CHROMagar™ STEC agar after 24, 48, and 72 h of incubation. The growth ratio, used as indicator of growth, was calculated by dividing the number of STEC colonies appearing on the plates after 24, 48, and 72 h by the number of the STEC colonies present in the cultures at time zero.

To determine growth of the STEC isolates in laboratory media without the impact of competing bacteria, each isolate was inoculated into 40 mL LB and incubated at 20 °C. For enumeration, appropriate dilutions of the cultures were plated on LB agar after 0 and 24 h. All experiments were performed in three biological replicates, except for STEC incubated in raw milk at 20 °C, which was only performed with two replicates. To exclude the presence of STEC in the two raw milk batches used, 6 × 100 µL raw milk samples from each batch were plated on CHROMagar™ STEC agar and incubated according to manufacturer's instructions.

2.2. Stx Production

A volume of 100 µL overnight LB-cultures was transferred to 5 mL fresh LB and incubated at 37 °C with agitation at 250 rpm until the optical density reached 0.5 at 600 nm (OD_{600}). Half of these cultures were induced by addition of 0.5 µg/mL of Mitomycin C (MMC). Both induced and uninduced cultures were incubated further for 3 h. Six samples,

three induced and thee uninduced, were processed and analyzed with respect to Stx content for each STEC isolate. The Stx content was measured in 1:20 dilutions of the cultures using the semi-quantitative enzyme immunoassay RIDASCREEN® Verotoxin kit (R-biopharm, Darmstadt, Germany) according to the manufacturer.

2.3. Genome Sequence Analyses

DNA for long-read sequencing was extracted using Nanobind CBB Big DNA Kit (NB-900-001-01, Circulomics, Baltimore, MD, USA), according to the manufacturer's instructions (Nanobind HMW DNA Extraction protocol for Gram-Negative Bacteria, 2021). Oxford Nanopore Technologies' "Ligation Sequencing kit" (SQK-LSK109, Oxford Nanopore Technologies Plc., Oxford, UK) was used for library preparation and "Native Barcoading Expansions" 1–12 (EXP-NBD104, Oxford Nanopore Technologies Plc., Oxford, UK) for barcoding the libraries. Nanopore sequencing was performed on a FLO-Min106 (R9.4.1, Oxford Nanopore Technologies Plc., Oxford, UK) flow cell. Recovered reads were assembled using the Flye assembler implemented in the "Dragonflye"-pipeline (https://github.com/rpetit3/dragonflye, v.1.0.12 (accessed on 25 March 2022)), which also performs adapter removal and assembly polishing. Virulence and antimicrobial resistance genes, core genome MLST type, and serotype were identified using the following tools on the CGE website: VirulenceFinder 2.0 [34,35], ResFinder 4.1 [36–38], cgMLSTFinder 1.1 [39,40], and SerotypeFinder 2.0 [41]. Prophage sequences were identified and annotated using the Phaster web software [42]. Isolate diversities were examined by SNP using Snippy v. 4.6.01 (https://github.com/tseemann/snippy (accessed on 20 May 2022)) and Mauve v2.4.0 (https://darlinglab.org/mauve/mauve.html (accessed on 5 May 2022)) were used to align the genomes (default parameters). This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession JANWGC000000000 to JANWGF000000000 (Table 1).

2.4. Statistics

For all growth experiments, a two-tailed paired Student's *t*-test, performed via Microsoft Office Excel, was used to test for statistically significant differences between average CFU determined at two different time points. *p*-values equal to or below 0.05 were considered significant. Standard deviation was calculated using Excel.

3. Results

3.1. Genetic Characterization of STEC Isolates from Raw Milk

Three of the four STEC isolates included in this study originated from the same farm (S2, S3, and S4); two were collected from fecal samples (S2 and S4) and one from an in-line milk filter sample (S3). Isolate S2 was collected five months prior to S3 and S4. The fourth isolate (S1) originates from a fecal sample from a second farm located within 10 km from farm one. The characteristics of the four STECs are listed in Table 1.

A genome sequence analysis revealed that isolates S1, S3, and S4 are highly similar and differ by only 19–23 SNPs, suggesting that these isolates are clonal (Figure 1).

S1, S3, and S4 exhibit 5.2 Mb chromosomes and the sequence analysis shows that they are of the serotype ONT:H28 and that they belong to the core genome multi-locus sequence type (cgMLST) 7679. Their genomes harbor the LEE-PAI-encoding intimin gamma (*eae*) and the gene encoding the translocated intimin receptor (Tir). The LEE-PAI is 99% identical over 33.3 kbp to the *E. coli* O157:H7 strain EDL933 (NCBI accession number NZ_CP008957) from the US outbreak in 1982 [4]. The lambdoid Stx1 phage of isolates S1, S3, and S4 is 99% identical over 22.8 kbp to Phage BP-4795 (*E. coli*, strain 4795/97, serotype O84:H4 human, Germany 1997) [15,43]. The CI repressor of this phage belongs to Clade V [11]. All three isolates carry a circular plasmid of 55 kbp encoding a heat-stable toxin (*astA*) and enterohaemolysin (*ehxA*) [44,45]. The heat-stable toxin is known to cause sporadic diarrhea in humans and animals [46], while enterohaemolysin is associated with bloody diarrhea and HUS [47]. Furthermore, in the genome of each isolate, a total of 18 prophages

of varying completeness were identified by Phaster [42,48]. The Stx phage harbored by these stains is of the lambdoid type and encodes Stx1a [4,49].

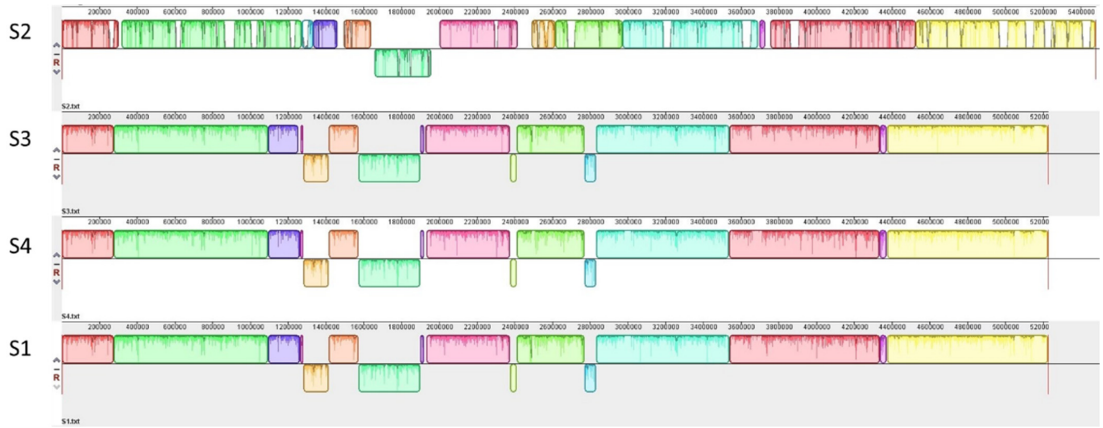

Figure 1. Multiple genome alignment was performed using the Mauve software. Each sequence is represented by a horizontal panel of blocks. The colored blocks indicate homologous sequence regions between the genomes. Blocks below the center line in each genome are inverted sequences with respect to the other genomes.

The genome of isolate S2 is highly different from those of S1, S3, and S4 (Figure 1). It comprises two circular contigs including a chromosome of 5.4 Mbp and a plasmid of 80 kbp. A DNA-typing analysis revealed that the isolate belongs to serotypes O108:H25 and cgMLST 141324. S2 carries a bacteriophage of Eru type 1 and a CI repressor belonging to Clade II [10,11]. The phage encodes the Stx2a type of Stx [50] and shares 99% identity over 22.2 kbp covering the replication region of the Stx2 phage TL-2011c (NCBI accession number NC_019442), which was carried by a highly virulent EHEC strain that caused an outbreak in Norway in 2006 [51].

Similar to the other three isolates, S2 harbors LEE-PAI including both *eae* and *tir*. The DNA sequence of the five LEE operons shows 87% identity over 30 kbp to the corresponding sequence of *E. coli* O157:H7 strain EDL933 (NCBI accession number NZ_CP008957). The 80 kbp plasmid of isolate S2 contains both *astA* and *ehxA*. Phaster identified 30 prophage regions on the chromosome and one prophage on the plasmid in isolate S2. ResFinder 4.1 did not detect antimicrobial resistance genes in any of the four isolates.

3.2. Stx Production

To explore the virulence potential of the STEC isolates, the Stx production was examined during growth in LB at 37 °C, with and without induction by MMC. All four isolates produced Stx and the levels were higher three hours post-induction with MMC compared to the uninduced samples (Figure 2).

3.3. Growth Characteristics of STEC Isolates in Raw Milk at Different Storage Temperatures

To examine the ability of the four STEC isolates to survive and grow in UPM, 40 mL raw milk samples were inoculated with approximately 3000–5000 CFU/mL of STEC culture. The samples were then incubated at 4 °C, 6 °C, 8 °C, and 20 °C for 72 h. After 0, 24, and 72 h, the samples were plated on Chromagar^{TM} STEC for enumeration. The growth ratios were calculated by dividing the number of STEC at 24 and 72 h by the number of bacteria inoculated into the milk.

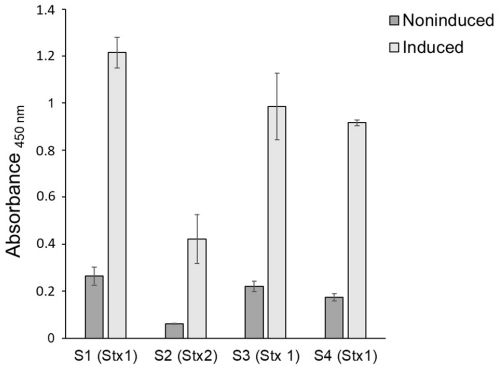

Figure 2. Semi-quantitative determination of Stx production of STEC isolates S1, S2, S3, and S4 after three hours induction with Mitomycin C (0.5 µg/mL). Error bars represent standard deviation.

At 4 °C, an average reduction in CFU (growth ratio below 1) was observed for all four isolates after storage for 24 h. The reduction was, however, not significant for any of the four isolates (Figure 3a). For isolates S1, S2, and S3, the number of CFU was further reduced over the next 48 h, while the level of isolate S4 remained unchanged (Figure 3a). The reduction in bacterial levels seen after 72 h, compared to the levels at the start of cultivation, was only significant for isolate S3 ($p < 0.01$). At 6 °C, a decrease in CFU/mL was observed during the first 24 h ($p \leq 0.05$ for isolate S1 and S4) but the cell death stopped after 24 h (Figure 3b). At 8 °C, S1, S3, and S4 multiplied over the first 24 h of storage (growth ratio above 1), and all strains showed increased CFU counts after 72 h (Figure 3c). The increase in CFU/mL after 72 h of storage, compared to the CFU at the start of cultivation, was significant only for isolate S1 ($p < 0.02$). There was a large difference in growth between isolate S2 and the three other isolates at 20 °C (Figure 3d).

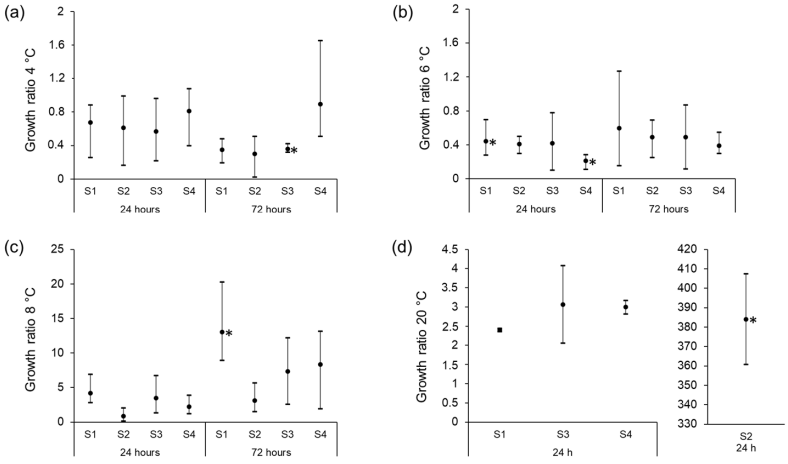

Figure 3. Chart showing the minimum, maximum, and average of growth ratios for STEC isolates S1, S2, S3, and S4 in unpasteurized milk at 4 °C (**a**), 6 °C (**b**), 8 °C (**c**), and 20 °C (**d**). Growth ratios below 1 indicate cell death while a growth ratio above 1 indicates growth. Asterisks represent statistical differences from pairwise comparisons between inoculation point and 24 or 72 h using two-tailed paired Student's *t* tests (* $p \leq 0.05$).

Under abused conditions, wherein the inoculated milk samples were kept at 4 °C but exposed to 20 °C for 90 min every 24 h, a trend of positive growth ratios was observed after 72 h of storage. However, only the increase in CFU/mL between 24 h and 48 h ($p < 0.01$) and between 24 h and 72 h ($p < 0.05$) for isolate S2 were significant (Figure 4a). The average growth ratios were lower than those observed at 8 °C (Figure 3c). The growth ratios of the four isolates inoculated into LB and incubated for 24 h indicate that the ability to grow in LB at 20 °C is similar for the four isolates (Figure 4b), and that they multiply faster in LB compared to unpasteurized milk at 20 °C.

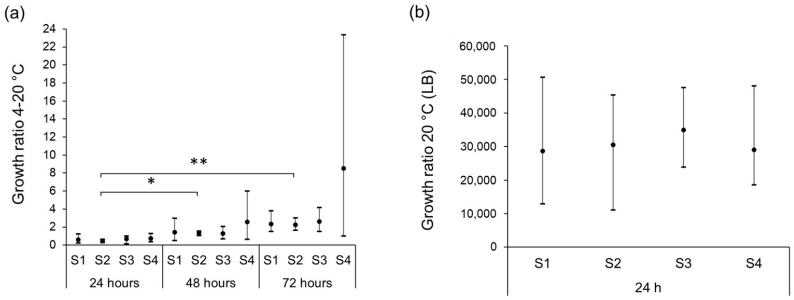

Figure 4. Chart showing the minimum, maximum, and average of growth ratios for STEC isolates S1, S2, S3, and S4 in unpasteurized milk at 4 °C under a temperature abuse scheme of 90 min at 20 °C every 24 h (**a**) and at 20 °C in LB-broth (**b**). Asterisks represent statistical differences from pairwise comparisons determined using two-tailed paired Student's *t* tests (* $p < 0.01$; ** $p < 0.05$).

Recordings of the temperature fluctuation in the 40 mL raw milk showed that after reaching 20 °C, it took >3 h for the milk to reach below 5 °C (Figure 5).

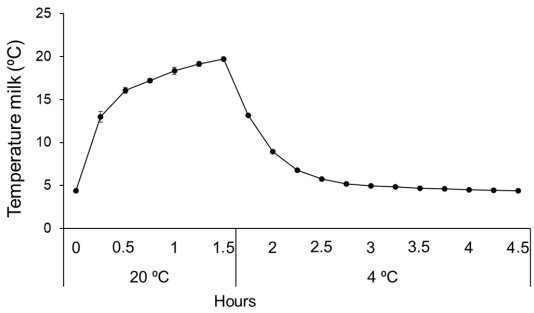

Figure 5. Temperature fluctuation in 40 mL UPM incubated at 4 °C, interrupted with incubation at 20 °C for 90 min. Error bars represent standard deviation.

4. Discussion

Cattle represent a reservoir of STEC, and the consumption of unpasteurized milk is, therefore, considered an important risk factor for contracting milk-borne STEC infections [1,2]. Herein, we explore the pathogenic potential of four *eae*-positive STECs (S1–S4) isolated from Norwegian dairy herds and their ability to grow in UPM stored under optimal and abused temperature conditions.

The genome analysis showed that isolates S1, S3, and S4 are clonal even though they were isolated from two different farms and S1 was isolated seven months prior to S3 and S4. This indicates that STEC has been transmitted between the two farms and persisted in the farm environment over time. Previous studies have shown that *E. coli* O157:H7 can survive for 99 days in soil [52] and 13 weeks in lake water at 15 °C [53]. The clonal isolates

S4 from feces and S3 from a milk filter were isolated the same day from the same farm, which strongly suggests that STEC can be transmitted from feces to the raw milk.

To explore the potential of the four isolates to cause disease, the genomes of the isolates were examined with respect to known virulence-associated genes. Isolates S1, S3, and S4 carry genes encoding Stx1a, while isolate S2 carries genes encoding Stx2a. Stx2a is considered the most potent Stx subtype and is associated with high virulence and HUS [50,54,55]. As isolate S2 has the potential to produce the more potent Stx2a form of Stx, it is likely to be more virulent than the other three isolates described in this study. All four isolates produced Stx, and the production was increased in the presence of MMC. In a study by Muniesa et al. (2004), 18% of 168 *stx2*-carrying STEC strains, isolated from cattle, were MMC-inducible [56]. Our results indicate a higher production of Stx1 by isolates S1, S2, and S4 compared to the degree of Stx2 production by isolate S2. The kit used for the detection of Stx, the enzyme immunoassay RIDASCREEN® Verotoxin kit (R-biopharm, Darmstadt, Germany), detects all known Stx-types [57]; however, a direct comparison between the amount of Stx1 and Stx2 produced is not applicable as the RIDASCREEN® Verotoxin kit has a lower detection limit for Stx1 (12.5 pg/mL) than for Stx2 (25 pg/mL). The degree of Stx production was examined at 37 °C, as this is the temperature in the human gut where the toxin's production occurs.

Stx-encoding prophages are very diverse and recent studies have suggested that their pathogenic potential is determined by the phage replication region, encoding the phage repressor protein CI and the phage replication proteins [10,11]. The EHEC phage replication unit Eru1, which is carried by the highly pathogenic EHEC strains that caused the Norwegian O103:H25 outbreak in 2006 and the large O104:H4 outbreak in Europe in 2011, is also carried by the S2 isolate described in this study [10]. Eru1 is often carried together with a Clade II CI repressor, as is the case for the S2 isolate, and may also indicate a high pathogenic potential [11]. It has previously been suggested that phage production is not induced by MMC in the Eru1 type of Stx-phages [10]. Contrary to this suggestion, we show herein that Stx production is induced by MMC in isolate S2, which suggests that Stx production and the production of new phage particles are regulated differently even in phages belonging to the same Eru type.

All four STEC isolates characterized in this study carry the gene encoding intimin, which has been associated with an increased ability to cause severe disease [55]. They also carry the large O157 plasmid harboring the virulence gene *ehxA*, encoding enterohaemolysin, which is present in most isolates from clinical STEC-infections [55]. The gene *astA*, encoding the heat-stable EAST1 toxin, which is present in several human diarrheagenic *E. coli* pathotypes was also found in in the genomes of the four isolates [46]. An EAST1-positive *E. coli* strain has been suggested to be the culprit of a large diarrhea outbreak in Japan that affected 2697 children [58]. The *astA* gene is, however, also commonly found among *E. coli* isolates collected from the environment [59]. The presence of genes encoding Stx, intimin, and enterohaemolysin as well as the EAST1 toxin in *E. coli* isolates from Norwegian dairy farms strongly indicate that Norwegian raw milk may contain highly pathogenic *E. coli*.

As raw milk may contain highly pathogenic bacteria such as STEC, *Listeria monocytogenes*, *Campylobacter,* and *Salmonella*, the temperature used for its storage is critical. In this study, we observed that at 4 °C the STEC levels slightly decreased over 72 h; however, only the reduction of S3 was significant ($p < 0.01$). At 6 °C, there was a trend towards decreased STEC levels over the first 24 h of storage, whereafter the levels were constant over the next 48 h. At 8 °C, there was an increasing trend in the STEC levels. Due to the large variation between the three biological replicates in the growth experiments, the results are not conclusive. However, at each temperature, at least one isolate showed a clear increase or decrease in CFU ($p \leq 0.05$), indicating that temperatures between 6 and 8 °C for more than 24 h may allow STEC to multiply in raw milk. These results are comparable to previous studies that have shown that *E. coli* O157:H7 is capable of growing in raw milk at 7 and 15 °C [60], but not at 5 °C [31]. Another study showed that *E. coli* O157:H7 did not decrease

during storage at 4 °C for five days. However, the study used streptomycin-resistant strains and raw milk supplemented with streptomycin, which may have influenced the natural microbiota of the raw milk [28]. The large growth variations between replicates of the same isolate in our study indicate that even though the growth is not statistically significant, sudden multiplications of STEC can occur in individual milk samples. The experimental conditions in the present study are not directly comparable to natural conditions since the raw milk was inoculated with 3000–5000 CFU/mL and such a high number of STEC is not likely to be present in fresh bulk tank milk. The transition from LB media at 37 °C—used for pre-culturing the isolates—to raw milk at low temperatures may also have influenced the survival of the isolates.

To mimic a real-life scenario of temperature abuse during meals, the milk was stored at 4 °C interrupted by exposure to room temperature (20 °C) for 1.5 h per day. Under these conditions, a general increase in CFU/mL milk was observed after 72 h; however, the increase was only significant for isolate S2 ($p < 0.05$). The recordings of the temperature in 40 mL of raw milk moved from 4 °C to 20 °C showed that the sample reached room temperature after 1.5 h. In a real-life situation, we assume that the volumes of raw milk stored for consumption are larger than 40 mL and the temperature fluctuation in the stored milk will be less pronounced.

Isolate S2 showed rapid growth during the storage of UPM at 20 °C, while the growth rates of the clonal isolates S1, S3, and S4 were slower. However, in LB media, all isolates showed similar growth rates and reached higher concentrations than they did in UPM, stored for the same time. The growth inhibition of the three clonal isolates may be due to the presence of milk-borne antimicrobial components such as lactoperoxidase, lysozyme, xanthine, oxidase, lactoferrin, immunoglobulins, and bacteriocins, or by competing microorganisms [61]. Previous studies have shown a better survival of *E. coli* inoculated in pasteurized milk compared to *E. coli* inoculated in UPM [31]. This is not surprising, since UPM contains an indigenous microbiota that can influence the growth of STEC. Notably, *E. coli* O157:H7 has been shown to be unresponsive to the antimicrobial activity of the lactoperoxidase–thiocyanate–hydrogen peroxide system (LPS) in milk, and this may also be the case for isolate S2 [60].

The survival and growth levels were only examined over a period of 72 h post-inoculation, as raw milk is not recommended to be stored for a very long time before consumption [62]. However, temperature abuse in consumers' handling practices is common both during transport and storage. Most consumers are unaware of their refrigerator's temperature [63], and studies show that household refrigerators often hold higher temperatures than recommended. Furthermore, milk is often kept at locations in the refrigerator where the temperature varies, for example, in refrigerator door racks [63–66]. This is particularly important to consider regarding the risk of disease from low-dose pathogens such as EHEC [67].

5. Conclusions

STEC isolates harboring genes associated with pathogenicity such as *stx1/2*, *eae*, *ehxA*, and *astA* are present in Norwegian dairy farms, and potentially pathogenic STEC isolates are able to can grow in raw milk stored at temperatures above 6 °C. As previous studies show that domestic refrigerators often hold higher temperatures than recommended, the growth of STEC in stored raw milk is a likely scenario. Altogether, the results suggest that UPM from Norwegian dairy farms may contain highly pathogenic STEC strains, and that the storage of UPM under suboptimal refrigeration conditions increases the risk for hemorrhagic colitis and HUS. To reduce the risk associated with the consumption of UPM, consumers need more knowledge regarding the importance of keeping the milk sufficiently chilled to prevent the growth and survival of STEC and other pathogenic bacteria. They should also be made aware of that even correctly stored UPM is associated with an increased risk for illness and that young children, elderly, and immunocompromised individuals are particularly vulnerable.

Author Contributions: Conceptualization, L.I., E.G.B.-G., M.A. and T.L.; methodology, L.I., E.G.B.-G., M.A. and T.L.; supervision, M.A., E.G.B.-G. and T.L.; writing—original draft preparation, L.I.; writing—review and editing, L.I., T.L., M.A. and E.G.B.-G. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: We thank Nofima and Helga Ness for the use of the Biosafety lab for performing the STEC storage experiment, and Anette Wold and Tove Maugesten for helpful instructions.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Karmali, M.A. Factors in the emergence of serious human infections associated with highly pathogenic strains of shiga toxin-producing *Escherichia coli*. *Int. J. Med. Microbiol.* **2018**, *308*, 1067–1072. [CrossRef] [PubMed]
2. Joseph, A.; Cointe, A.; Mariani-Kurkdjian, P.; Rafat, C.; Hertig, A. Shiga toxin-associated hemolytic uremic syndrome: A narrative review. *Toxins* **2020**, *12*, 67. [CrossRef] [PubMed]
3. European Food Safety Authority & European Centre for Disease Prevention and Control. The European Union One Health 2020 Zoonoses Report. *EFSA J.* **2021**, *19*, e06971. [CrossRef]
4. Riley, L.W.; Remis, R.S.; Helgerson, S.D.; McGee, H.B.; Wells, J.G.; Davis, B.R.; Hebert, R.J.; Olcott, E.S.; Johnson, L.M.; Hargrett, N.T. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. *N. Engl. J. Med.* **1983**, *308*, 681–685. [CrossRef]
5. Gould, L.H.; Mody, R.K.; Ong, K.L.; Clogher, P.; Cronquist, A.B.; Garman, K.N.; Latrop, S.; Medus, C.; Spina, N.L.; Webb, T.H. Increased recognition of non-O157 Shiga toxin–producing *Escherichia coli* infections in the United States during 2000–2010: Epidemiologic features and comparison with *E. coli* O157 infections. *Foodborne Pathog. Dis.* **2013**, *10*, 453–460. [CrossRef]
6. Hedican, E.B.; Medus, C.; Besser, J.M.; Juni, B.A.; Koziol, B.; Taylor, C.; Smith, K.E. Characteristics of O157 versus non-O157 Shiga toxin-producing *Escherichia coli* infections in Minnesota, 2000–2006. *Clin. Infect. Dis.* **2009**, *49*, 358–364. [CrossRef]
7. Hadler, J.L.; Clogher, P.; Hurd, S.; Phan, Q.; Mandour, M.; Bemis, K.; Marcus, R. Ten-year trends and risk factors for non-O157 Shiga toxin–producing *Escherichia coli* found through Shiga toxin testing, Connecticut, 2000–2009. *Clin. Infect. Dis.* **2011**, *53*, 269–276. [CrossRef]
8. Tuttle, J.; Gomez, T.; Doyle, M.; Wells, J.; Zhao, T.; Tauxe, R.; Griffin, P.M. Lessons from a large outbreak of *Escherichia coli* O157:H7 infections: Insights into the infectious dose and method of widespread contamination of hamburger patties. *Epidemiol. Infect.* **1999**, *122*, 185–192. [CrossRef]
9. Sperandio, V.; Nguyen, Y. Enterohemorrhagic *E. coli* (EHEC) pathogenesis. *Front. Cell. Infect. Microbiol.* **2012**, *2*, 90. [CrossRef]
10. Llarena, A.-K.; Aspholm, M.; O'Sullivan, K.; Wêgrzyn, G.; Lindbäck, T. Replication region analysis reveals non-lambdoid shiga toxin converting bacteriophages. *Front. Microbiol.* **2021**, *12*, 640945. [CrossRef]
11. Fagerlund, A.; Aspholm, M.; Węgrzyn, G.; Lindbäck, T. High diversity in the regulatory region of Shiga toxin encoding bacteriophages. *BMC Genom.* **2022**, *23*, 230. [CrossRef] [PubMed]
12. Bednarz, M.; Halliday, J.A.; Herman, C.; Golding, I. Revisiting bistability in the lysis/lysogeny circuit of bacteriophage lambda. *PLoS ONE* **2014**, *9*, e100876. [CrossRef] [PubMed]
13. Casjens, S.R.; Hendrix, R.W. Bacteriophage lambda: Early pioneer and still relevant. *Virology* **2015**, *479*, 310–330. [CrossRef] [PubMed]
14. Zeng, L.; Skinner, S.O.; Zong, C.; Sippy, J.; Feiss, M.; Golding, I. Decision making at a subcellular level determines the outcome of bacteriophage infection. *Cell* **2010**, *141*, 682–691. [CrossRef] [PubMed]
15. Zhang, W.; Kohler, B.; Oswald, E.; Beutin, L.; Karch, H.; Morabito, S.; Caprioli, A.; Suerbaum, S.; Schmidt, H. Genetic diversity of intimin genes of attaching and effacing *Escherichia coli* strains. *J. Clin. Microbiol.* **2002**, *40*, 4486–4492. [CrossRef]
16. Yang, X.; Sun, H.; Fan, R.; Fu, S.; Zhang, J.; Matussek, A.; Xiong, Y.; Bai, X. Genetic diversity of the intimin gene (*eae*) in non-O157 Shiga toxin-producing *Escherichia coli* strains in China. *Sci. Rep.* **2020**, *10*, 3275. [CrossRef]
17. De Rauw, K.; Buyl, R.; Jacquinet, S.; Piérard, D. Risk determinants for the development of typical haemolytic uremic syndrome in Belgium and proposition of a new virulence typing algorithm for Shiga toxin-producing *Escherichia coli*. *Epidemiol. Infect.* **2019**, *147*, E6. [CrossRef]
18. European Food Safety Authority & European Centre for Disease Prevention Control. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2009. *EFSA J.* **2011**, *9*, 2090. [CrossRef]
19. Bosilevac, J.M.; Koohmaraie, M. Prevalence and characterization of non-O157 Shiga toxin-producing *Escherichia coli* isolates from commercial ground beef in the United States. *Appl. Environ. Microbiol.* **2011**, *77*, 2103–2112. [CrossRef]

20. European Food Safety Authority. Scientific Opinion of the Panel on Biological Hazards (BIOHAZ)-Monitoring of verotoxigenic *Escherichia coli* (VTEC) and identification of human pathogenic VTEC types. *EFSA J.* **2007**, *5*, 579. [CrossRef]
21. Gillespie, I.; Adak, G.; O'brien, S.; Bolton, F. Milkborne general outbreaks of infectious intestinal disease, England and Wales, 1992–2000. *Epidemiol. Infect.* **2003**, *130*, 461–468. [CrossRef] [PubMed]
22. Ballem, A.; Gonçalves, S.; Garcia-Meniño, I.; Flament-Simon, S.C.; Blanco, J.E.; Fernandes, C.; Saavedra, M.J.; Pinto, C.; Oliveira, H.; Blanco, J.; et al. Prevalence and serotypes of Shiga toxin-producing *Escherichia coli* (STEC) in dairy cattle from Northern Portugal. *PLoS ONE* **2021**, *15*, e0244713. [CrossRef] [PubMed]
23. Venegas-Vargas, C.; Henderson, S.; Khare, A.; Mosci, R.E.; Lehnert, J.D.; Singh, P.; Ouellette, L.M.; Norby, B.; Funk, J.A.; Rust, S. Factors associated with Shiga toxin-producing *Escherichia coli* shedding by dairy and beef cattle. *Appl. Environ. Microbiol.* **2016**, *82*, 5049–5056. [CrossRef] [PubMed]
24. Jaakkonen, A.; Castro, H.; Hallanvuo, S.; Ranta, J.; Rossi, M.; Isidro, J.; Lindström, M.; Hakkinen, M. Longitudinal Study of Shiga Toxin-Producing *Escherichia coli* and *Campylobacter jejuni* on Finnish Dairy Farms and in Raw Milk. *Appl. Environ. Microbiol.* **2019**, *85*, e02910-18. [CrossRef]
25. Idland, L.; Granquist, E.G.; Aspholm, M.; Lindbäck, T. The prevalence 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. *J. Appl. Microbiol.* **2022**, *132*, 3959–3972. [CrossRef]
26. Geue, L.; Segura-Alvarez, M.; Conraths, F.; Kuczius, T.; Bockemühl, J.; Karch, H.; Gallien, P. A long-term study on the prevalence of Shiga toxin-producing *Escherichia coli* (STEC) on four German cattle farms. *Epidemiol. Infect.* **2002**, *129*, 173–185. [CrossRef]
27. D'aoust, J.-Y.; Park, C.; Szabo, R.; Todd, E.; Emmons, D.; McKellar, R. Thermal inactivation of *Campylobacter* species, *Yersinia enterocolitica*, and hemorrhagic *Escherichia coli* 0157: H7 in fluid milk. *J. Dairy Sci.* **1988**, *71*, 3230–3236. [CrossRef]
28. Leclair, R.M.; McLean, S.K.; Dunn, L.A.; Meyer, D.; Palombo, E.A. Investigating the effects of time and temperature on the growth of *Escherichia coli* O157: H7 and *Listeria monocytogenes* in raw cow's milk based on simulated consumer food handling practices. *Int. J. Environ. Res. Public Health* **2019**, *16*, 2691. [CrossRef]
29. Kauppi, K.; Tatini, S.; Harrell, F.; Feng, P. Influence of substrate and low temperature on growth and survival of verotoxigenic *Escherichia coli*. *Food Microbiol.* **1996**, *13*, 397–405. [CrossRef]
30. International Commission on Microbiological Specifications for Foods. *Microorganisms in Foods 5: Characteristics of Microbial Pathogens*, 1st ed.; Kluwer Academic/Plenum Publishers: London, UK, 1996.
31. Wang, G.; Zhao, T.; Doyle, M.P. Survival and growth of *Escherichia coli* O157: H7 in unpasteurized and pasteurized milk. *J. Food Prot.* **1997**, *60*, 610–613. [CrossRef]
32. Dumitraşcu, L.; Nicolau, A.I.; Neagu, C.; Didier, P.; Maître, I.; Nguyen-The, C.; Skuland, S.E.; Møretrø, T.; Langsrud, S.; Truninger, M. Time-temperature profiles and *Listeria monocytogenes* presence in refrigerators from households with vulnerable consumers. *Food Control* **2020**, *111*, 107078. [CrossRef]
33. Roccato, A.; Uyttendaele, M.; Membré, J.-M. Analysis of domestic refrigerator temperatures and home storage time distributions for shelf-life studies and food safety risk assessment. *Food Res. Int.* **2017**, *96*, 171–181. [CrossRef] [PubMed]
34. Joensen, K.G.; Scheutz, F.; Lund, O.; Hasman, H.; Kaas, R.S.; Nielsen, E.M.; Aarestrup, F.M. Real-time whole-genome sequencing for routine typing, surveillance, and outbreak detection of verotoxigenic *Escherichia coli*. *J. Clin. Microbiol.* **2014**, *52*, 1501–1510. [CrossRef]
35. Malberg Tetzschner, A.M.; Johnson, J.R.; Johnston, B.D.; Lund, O.; Scheutz, F. In silico genotyping of *Escherichia coli* isolates for extraintestinal virulence genes by use of whole-genome sequencing data. *J. Clin. Microbiol.* **2020**, *58*, e01269-20. [CrossRef] [PubMed]
36. Camacho, C.; Coulouris, G.; Avagyan, V.; Ma, N.; Papadopoulos, J.; Bealer, K.; Madden, T.L. BLAST+: Architecture and applications. *BMC Bioinform.* **2009**, *10*, 421. [CrossRef]
37. Bortolaia, V.; Kaas, R.S.; Ruppe, E.; Roberts, M.C.; Schwarz, S.; Cattoir, V.; Philippon, A.; Allesoe, R.L.; Rebelo, A.R.; Florensa, A.F. ResFinder 4.0 for predictions of phenotypes from genotypes. *J. Antimicrob. Chemother.* **2020**, *75*, 3491–3500. [CrossRef]
38. Zankari, E.; Allesøe, R.; Joensen, K.G.; Cavaco, L.M.; Lund, O.; Aarestrup, F.M. PointFinder: A novel web tool for WGS-based detection of antimicrobial resistance associated with chromosomal point mutations in bacterial pathogens. *J. Antimicrob. Chemother.* **2017**, *72*, 2764–2768. [CrossRef]
39. Clausen, P.T.; Aarestrup, F.M.; Lund, O. Rapid and precise alignment of raw reads against redundant databases with KMA. *BMC Bioinform.* **2018**, *19*, 307. [CrossRef]
40. Zhou, Z.; Alikhan, N.-F.; Mohamed, K.; Fan, Y.; Achtman, M.; Brown, D.; Chattaway, M.; Dallman, T.; Delahay, R.; Kornschober, C. The EnteroBase user's guide, with case studies on *Salmonella* transmissions, *Yersinia pestis* phylogeny, and *Escherichia* core genomic diversity. *Genome Res.* **2020**, *30*, 138–152. [CrossRef]
41. Joensen, K.G.; Tetzschner, A.M.M.; Iguchi, A.; Aarestrup, F.M.; Scheutz, F. Rapid and easy in silico serotyping of *Escherichia coli* isolates by use of whole-genome sequencing data. *J. Clin. Microbiol.* **2015**, *53*, 2410–2426. [CrossRef]
42. Arndt, D.; Grant, J.R.; Marcu, A.; Sajed, T.; Pon, A.; Liang, Y.; Wishart, D.S. PHASTER: A better, faster version of the PHAST phage search tool. *Nucleic Acids Res.* **2016**, *44*, W16–W21. [CrossRef] [PubMed]
43. Creuzburg, K.; Recktenwald, J.R.; Kuhle, V.; Herold, S.; Hensel, M.; Schmidt, H. The Shiga toxin 1-converting bacteriophage BP-4795 encodes an NleA-like type III effector protein. *J. Bacteriol. Parasitol.* **2005**, *187*, 8494–8498. [CrossRef] [PubMed]

44. Savarino, S.J.; McVeigh, A.; Watson, J.; Cravioto, A.; Molina, J.; Echeverria, P.; Bhan, M.K.; Levine, M.M.; Fasano, A. Enteroaggregative *Escherichia coli* heat-stable enterotoxin is not restricted to enteroaggregative *E. coli*. *J. Infect. Dis.* **1996**, *173*, 1019–1022. [CrossRef] [PubMed]
45. Lorenz, S.C.; Son, I.; Maounounen-Laasri, A.; Lin, A.; Fischer, M.; Kase, J.A. Prevalence of hemolysin genes and comparison of *ehxA* subtype patterns in Shiga toxin-producing *Escherichia coli* (STEC) and non-STEC strains from clinical, food, and animal sources. *Appl. Environ. Microbiol.* **2013**, *79*, 6301–6311. [CrossRef] [PubMed]
46. Veilleux, S.; Dubreuil, J.D. Presence of *Escherichia coli* carrying the EAST1 toxin gene in farm animals. *Vet. Res.* **2006**, *37*, 3–13. [CrossRef]
47. Hua, Y.; Zhang, J.; Jernberg, C.; Chromek, M.; Hansson, S.; Frykman, A.; Xiong, Y.; Wan, C.; Matussek, A.; Bai, X. Molecular Characterization of the Enterohemolysin Gene (*ehxA*) in Clinical Shiga Toxin-Producing *Escherichia coli* Isolates. *Toxins* **2021**, *13*, 71. [CrossRef]
48. Zhou, Y.; Liang, Y.; Lynch, K.; Dennis, J.; Wishart, D. PHAST: A fast phage search tool. *Nucleic Acids Res.* **2011**, *39*, W347–W352. [CrossRef]
49. Melton-Celsa, A.R. Shiga toxin (Stx) classification, structure, and function. *Microbiol. Spectr.* **2014**, *2*, 6. [CrossRef]
50. Fuller, C.A.; Pellino, C.A.; Flagler, M.J.; Strasser, J.E.; Weiss, A.A. Shiga toxin subtypes display dramatic differences in potency. *Infect. Immun.* **2011**, *79*, 1329–1337. [CrossRef]
51. L'Abée-Lund, T.M.; Jørgensen, H.J.; O'Sullivan, K.; Bohlin, J.; Ligård, G.; Granum, P.E.; Lindbäck, T. The highly virulent 2006 Norwegian EHEC O103: H25 outbreak strain is related to the 2011 German O104: H4 outbreak strain. *PLoS ONE* **2012**, *7*, e31413. [CrossRef]
52. Bolton, D.; Byrne, C.; Sheridan, J.; McDowell, D.; Blair, I. The survival characteristics of a non-toxigenic strain of *Escherichia coli* O157: H7. *J. Appl. Microbiol.* **1999**, *86*, 407–411. [CrossRef] [PubMed]
53. Wang, G.; Doyle, M.P. Survival of enterohemorrhagic *Escherichia coli* O157: H7 in water. *J. Food Prot.* **1998**, *61*, 662–667. [CrossRef] [PubMed]
54. Krüger, A.; Lucchesi, P.M. Shiga toxins and stx phages: Highly diverse entities. *Microbiology* **2015**, *161*, 451–462. [CrossRef] [PubMed]
55. Brandal, L.T.; Wester, A.L.; Lange, H.; Løbersli, I.; Lindstedt, B.-A.; Vold, L.; Kapperud, G. Shiga toxin-producing *Escherichia coli* infections in Norway, 1992-2012: Characterization of isolates and identification of risk factors for haemolytic uremic syndrome. *BMC Infect. Dis.* **2015**, *15*, 324. [CrossRef]
56. Muniesa, M.; Blanco, J.E.; De Simón, M.; Serra-Moreno, R.; Blanch, A.R.; Jofre, J. Diversity of *stx2* converting bacteriophages induced from Shiga-toxin-producing *Escherichia coli* strains isolated from cattle. *Microbiology* **2004**, *150*, 2959–2971. [CrossRef]
57. Beutin, L.; Steinrück, H.; Krause, G.; Steege, K.; Haby, S.; Hultsch, G.; Appel, B. Comparative evaluation of the Ridascreen® Verotoxin enzyme immunoassay for detection of Shiga-toxin producing strains of *Escherichia coli* (STEC) from food and other sources. *J. Appl. Microbiol.* **2007**, *102*, 630–639. [CrossRef]
58. Itoh, Y.; Nagano, I.; Kunishima, M.; Ezaki, T. Laboratory investigation of enteroaggregative *Escherichia coli* O untypeable: H10 associated with a massive outbreak of gastrointestinal illness. *J. Clin. Microbiol.* **1997**, *35*, 2546–2550. [CrossRef]
59. Sidhu, J.P.; Ahmed, W.; Hodgers, L.; Toze, S. Occurrence of virulence genes associated with diarrheagenic pathotypes in *Escherichia coli* isolates from surface water. *Appl. Environ. Microbiol.* **2013**, *79*, 328–335. [CrossRef]
60. Heuvelink, A.; Bleumink, B.; Van Den Biggelaar, F.; Te Giffel, M.; Beumer, R.; De Boer, E. Occurrence and survival of verocytotoxin-producing *Escherichia coli* O157 in raw cow's milk in The Netherlands. *J. Food Prot.* **1998**, *61*, 1597–1601. [CrossRef]
61. Claeys, W.L.; Cardoen, S.; Daube, G.; De Block, J.; Dewettinck, K.; Dierick, K.; De Zutter, L.; Huyghebaert, A.; Imberechts, H.; Thiange, P. Raw or heated cow milk consumption: Review of risks and benefits. *Food Control* **2013**, *31*, 251–262. [CrossRef]
62. European Food Safety Authority, panel on Biological Hazards. Scientific opinion on the public health risks related to the consumption of raw drinking milk. *EFSA J.* **2015**, *13*, 3940. [CrossRef]
63. Marklinder, I.; Lindblad, M.; Eriksson, L.; Finnson, A.; Lindqvist, R. Home storage temperatures and consumer handling of refrigerated foods in Sweden. *J. Food Prot.* **2004**, *67*, 2570–2577. [CrossRef] [PubMed]
64. Evans, E.W.; Redmond, E.C. Time-temperature profiling of United Kingdom consumers' domestic refrigerators. *J. Food Prot.* **2016**, *79*, 2119–2127. [CrossRef] [PubMed]
65. Kennedy, J.; Jackson, V.; Blair, I.; McDowell, D.; Cowan, C.; Bolton, D. Food safety knowledge of consumers and the microbiological and temperature status of their refrigerators. *J. Food Prot.* **2005**, *68*, 1421–1430. [CrossRef] [PubMed]
66. Koutsoumanis, K.; Pavlis, A.; Nychas, G.-J.E.; Xanthiakos, K. Probabilistic model for *Listeria monocytogenes* growth during distribution, retail storage, and domestic storage of pasteurized milk. *Appl. Environ. Microbiol.* **2010**, *76*, 2181–2191. [CrossRef]
67. Zech, H.; Echtermeyer, C.; Wöhlbrand, L.; Blasius, B.; Rabus, R. Biological versus technical variability in 2-D DIGE experiments with environmental bacteria. *Proteomics* **2011**, *11*, 3380–3389. [CrossRef]

ISBN: 978-82-575-2024-3
ISSN: 1894-6402

Norwegian University
of Life Sciences

Postboks 5003
NO-1432 Ås, Norway
+47 67 23 00 00
www.nmbu.no

115992 / ANDVORDGRAFISK.NO