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Coxiella burnetii DNA in milk, milk products, and fermented dairy products

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Abstract

Introduction: Q fever in dairy cattle has been investigated in Latvia since 2012. In 2015, 10.7% of farms tested positive for the DNA of *C. burnetii*, its aetiological agent, in bulk tank milk. The presence of *C. burnetii* DNA and infectious bacteria in dairy products has been assessed in several countries, and because Latvian milk may contain them, parallel assessment in this country is recommended. Accordingly, the present study tested shop and farm retail dairy products from Latvia and included foreign products for comparison. **Material and Methods:** Investigation was carried out of 187 samples of a diverse range of dairy products from 41 Latvian milk producers. Twenty-six comparable samples pooled from Estonia, France, Germany, Greece, Italy, Lithuania, the Netherlands, Poland and Spain were also included. The all-countries total number of fermented milk products was 160. Special attention was paid to products that could be more attractive to children because of their added chocolate, cacao, berry and fruit content. DNA was extracted and amplification of *C. burnetii IS1111* was performed using a commercial PCR kit. **Results:** Overall positivity was 60.56%. Domestic products were positive more often (60.96%) than foreign ones (57.69%). Only 26.67% of unpasteurised Latvian cow's milk samples were positive whereas 76.47% of pasteurised equivalents and 63.13% of fermented milk products were. Sweetened and fruit-containing samples were 71.43% positive. **Conclusion:** The shedding of *C. burnetii via* milk should be monitored and only milk from healthy animals allowed for sale for direct human consumption without pasteurisation. Raw milk quality and the effectiveness of industrial heat treatment and pasteurisation methods in Latvia and other countries should be carefully assessed to ensure adequate consumer health protection.

Keywords: Q fever, *Coxiella burnetii*, dairy products, zoonosis.

Introduction

The zoonotic disease Q fever is widespread globally, and poses a serious threat to human health in Europe. The disease-causing agent is *Coxiella burnetii*, a mandatory intracellular pathogen. These bacteria enter the organism *via* aerosols, arthropod vectors or alimentary route, attach to the host macrophages, and enter them. *C. burnetii* creates a specific structure inside the cell by phagocytosis, the parasitic vacuole, and thus remains viable (27).

In the period of 2015–2019, the total number of confirmed human cases of Q fever in the European Union ranged from 822 to 950 per year, corresponding to 0.19 cases per 100,000 population (13). One to three human cases per year were registered in Latvia during the years 2008–2015. Human cases of Q fever were not reported during the more recent period of 2016–2019; however, one case was identified in the first quarter of 2020 (8).

Few data on human Q fever infections conclusively proven to have been *via* contaminated unpasteurised milk and dairy products have been published. Contaminated home-made cheese was indicated only as a possible source of human infections in Canada during an outbreak among goat farmers or farm workers (n = 146), and in sporadic paediatric cases in Greece (n = 8), the infected persons were also in contact with animals as farm workers or were visiting rural areas (21, 25). However, while the possibility of non-alimentary route infections existed in the latter case, statistical significance–indicating P values emerged for unpasteurised dairy product consumption against other risk factors. Several consumers (n = 5) of raw cow's milk from

a common dairy were infected by *C. burnetii* in the USA (37), and consumption of raw milk was indicated as a significant factor for infected Colombian farmers (6). Ingestion of contaminated raw milk or dairy products may often lead to seroconversion but rarely causes clinical Q fever (9). Nevertheless, food consumption cannot be excluded from the assessment of Q fever transmission routes, and a One Health approach must extend to consideration of this possibility; however, food has been regarded as a "seldom recorded route" for the transmission of this pathogen (29, 23). A comprehensive literature review in 2018 (32) and simulation studies (16) concluded that the risk of *C. burnetii* human infection due to consumption of unpasteurised milk and raw milk products "cannot be considered negligible".

In some European countries, shedding of this pathogen *via* cattle milk has been recorded frequently, an example being its detection in 31.54% of tested dairy cattle herds' milk in Poland (40). A survey of dairy cattle operations in Latvia showed that 10.7% tested positive for the presence of *C. burnetii* DNA in bulk tank milk samples (3).

Dairy products besides milk have been tested for the presence of *C. burnetii* DNA in several countries. In Poland, it was found that 69.2% of such products were positive in tests (40). In Spain, *C. burnetii* DNA was detected in 29.9% of hard cheeses produced from raw sheep's milk and 7.6% contained infectious bacteria (1). In France, 64% of tested dairy products contained the DNA of this pathogen, but none contained viable bacteria (11).

C. burnetii serves as the target organism for proving the effectiveness of milk pasteurisation under the recommended conditions of 63°C for 30 min or 72°C for 15 s (9), but treatment at ultra-high temperature (UHT) of 135°C can be also used for milk according to Commission Regulation (EC) No 1662/2006 of 6 November 2006. It is assumed that pasteurisation should kill the Q fever agent in raw milk (12, 22). The thermal resistance parameters of *C. burnetii* are a D value of 3.73 min at 63°C and a z value of 4.34°C. The suggested pasteurisation conditions are reported to achieve a reduction of from 4.7 to 8 orders of magnitude in viable *C. burnetii* cells (9).

In the production of fermented sour milk products (yogurts, yogurt drinks and kefir), one of two pasteurisation regimes can be used: 85–87°C for 5–10 min or 90–95°C for 2–8 min. Sour cream is obtained from cream after milk separation and pasteurisation at 84–88°C for 2–10 min or 85–98°C for 20 s, depending on the fat content, and after treatment with lactic acid bacteria cultures. A homogenisation step can also be used at 50–70°C and 6–12.5 MPa. During the cottage cheese production process, the pasteurisation is carried out at 76–80°C for 15–20 s. In Latvia, the lowest pasteurisation temperatures are used for the raw milk in the cheese production process: 72–76°C for 15–20 s (29). Goat's milk is usually pasteurised at 72°C for 15 s (5).

There are around 40 milk processing companies operating in Latvia (18), but raw milk can be sold directly to consumers as well. For milk sold unprocessed, the current microbiological quality requirements according to Regulation No. 73 of the Cabinet of Ministers of Latvia "The requirements for small-scale circulation of raw cow's and goat's milk" (for production on a scale of less than 1000 t per year) are as follows: the total number of bacteria at a temperature of 30°C must be $\leq 100{,}000$ mL^{-1}, the number of somatic cells $\leq 400{,}000$ mL^{-1}, the number of *Staphylococcus aureus* colony-forming units ≤ 500 mL^{-1}, and *Salmonella* spp. must not be present in 25 mL of milk. According to Regulation No. 597 of the Cabinet of Ministers of Latvia on "Veterinary, hygiene, and safety requirements for the circulation of raw milk" and Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for foods of animal origin, raw milk must come from a herd officially free of brucellosis and tuberculosis and from healthy animals without any sign of infectious diseases, without genital tract infections or inflammation of the udder. The criteria for the total number of bacteria at a temperature of 30°C and the number of somatic cells are the same as stipulated for small-scale circulation in the pertinent Latvian regulation. According to the Commission regulation, for raw milk from other species than cows, the total number of bacteria at a temperature of 30°C must be $\leq 1{,}500{,}000$ mL^{-1} but if the milk is intended for the manufacture of products made with raw milk without any heat treatment, this quality attribute's value must be below 500,000 mL^{-1}. Mandatory sample testing is to take place at least twice a month. If the herd does not have official status free of brucellosis and tuberculosis but the animals are healthy, the raw milk can be used for the production of cheese with a maturation period of at least two months (Latvian Regulation No. 597). According to both the Commission and Latvian regulations, the presence of *C. burnetii* DNA does not need to be monitored. In a study from the USA, it was shown that the presence of *C. burnetii* DNA in milk strongly correlates with the somatic cell count (2).

In summary, the quality of milk and milk products depends on the combination of various factors, namely animal health, milk microbiological quality, and the technological processes of food production. Assuming that raw milk must be obtained only from healthy animals and its quality is compliant with requirements of the Latvian and European Union legislation, whether these stipulations as the existing quality criteria are sufficient to exclude the presence of *C. burnetii* DNA in the food chain is the question which this study aimed to resolve.

Taking into account that paediatric cases of alimentary infection with Q fever have been described in some countries (25) and dairy products are an important part of the human diet in Latvia from as early as six months of age (39), special attention was

paid to yogurts and other fermented products with added ingredients, which are often consumed by children as well as adults (24, 38). The present study focused on unpasteurised and pasteurised milk samples and fermented dairy products with added ingredients from the retail market in Latvia and assessed the presence of *C. burnetii* DNA in them.

Material and Methods

Samples and sampling. Samples were obtained from the largest supermarket chains of Latvia, from the Central Market of Riga or directly from the producers. Unpasteurised bulk milk samples in retail trade were collected in sterile milk sampling containers. Other samples were prefilled by producers in milk cartons, plastic or glass milk jars or other packaging designed for the particular product type. After the purchase of the samples, they were immediately transported to the laboratory in a cold box at 4 to 6°C and processed or stored for one to three days in a refrigerator at the same temperature.

The total number of samples tested was 213. The 187 samples which were from Latvia originated from 41 producers: milk processing companies, individual farms or artisanal producers. Thirty-two samples represented 18 individual cattle or goat farms selling raw milk and milk products. The herd size of the cattle farms ranged from 5 to 500 cows, and that of the goat farms ranged from 100 to 300 animals. Milk processing companies were the origin of 155 samples: 17 pasteurised milk samples with fat content of 1.5–4.0% as indicated on the label, 28 yogurt and yogurt drink samples (mainly with fruit, berries and other ingredients), 40 cottage cheese and home-style cheese samples made from skimmed or whole milk with fat content of 0.5 or 9.0%, respectively, and 68 other cow's milk products. Among the 41 domestic producers sampled, the four largest Latvian dairy processing companies were represented with 10 to 31 samples each. Twenty-six samples originating from other countries (Estonia (1), France (1), Germany (4), Greece (1), Italy (1), Lithuania (10), the Netherlands (2), Poland (5), and Spain (1)) were also included in the study. Nine samples were UHT products: three were cream, three were protein drinks, two goat's milk, and one pasteurised milk.

Sixty four fermented products and three pasteurised milk samples (in total n = 67) contained various added ingredients for flavour. Special attention was paid to products that could be more attractive to children with chocolate, cacao, berries and other fruit (n = 56). Dill, garlic, dried garlic, dried onions, leeks, parsley, natural herbal flavouring, wheat sprouts, paprika, seed mixture, spices and ham were also constituents of some fermented milk products tested (n = 11). The total number of fermented products was 160. The sample size variation and other information are given in Table 1.

DNA extraction and real time PCR. Solid samples were cut into small pieces using a sterile scalpel. Total DNA was extracted from 200 µL or 100 mg of sample with a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) adopting a 100 µL elution volume.

Table 1. The overview of the analysed samples

Product type	Producers	Number of samples originating from Latvia/those with additives	Number of samples from foreign sources/those with additives	Sample size
Cow's milk products				
Unpasteurised milk	Individual farms	15/0	0/0	20–1,000 mL
Heat-treated milk	Milk processing companies	3/0	0/0	500–1,000 mL
Pasteurised milk	Milk processing companies	17/3	2/0	200–1,000 mL
Yogurts, yogurt drinks	Milk processing companies, individual farms	28/23	4/4	100–700 g
Cottage cheese, home-style cheese, and desserts	Milk processing companies	40/17	4/2	35–400 g
Cheese (hard, semi-hard, smoked, and cheese spread)	Milk processing companies	23/6	2/0	30–342 g
Cream, coffee cream, and sour cream	Milk processing companies, individual farms	27 (2[a])/0	2[a]/0	100–600 g or 20– 200 mL
Kefir, ryazhenka, pure culture fermented milk, buttermilk, and sour milk	Milk processing companies	19/4	3/2	150–1,000 g
Protein drink	Brewery, milk processing companies	6 (3[a])/5	0/0	250–460 mL
Goat's milk products				
Pasteurised goat's milk	Milk processing companies, individual farms	1/0	2[a]/0	500–1,000 mL
Goat's cheese	Milk processing companies, individual farms	8/1	7/0	100–200 g
TOTAL		187/59	26/8	

[a] – produced using ultra-high-temperature treatment technology

Table 2. Summary of results for all samples tested

Product type	Number of samples from Latvian producers (positive)	Percentage of positive samples	Average Ct of positive samples	Number of samples from foreign producers (positive)	Percentage of positive samples	Average Ct of positive samples
Unpasteurised milk	15 (4)	26.67	30.45	0	-	-
Heat-treated milk	3 (0)	0.00	-	0	-	-
Pasteurised milk	17 (13)	76.47	30.41	2 (2)	100.00	34.72
Yogurts, yogurt drinks	28 (15)	53.57	31.75	4 (2)	50.00	33.99
Cottage cheese, home cheese, and desserts	40 (31)	77.50	31.08	4 (3)	75.00	29.65
Cheese	23 (20)	86.96	29.84	2 (2)	100.00	28.40
Cream, coffee cream, and sour cream	27 (15)	55.56	34.06	2 (1)	50.00	35.07
Kefir, ryazhenka, pure culture fermented milk, buttermilk, sour milk	19 (11)	57.89	32.97	3 (2)	66.67	33.33
Protein drink	6 (5)	83.33	34.21	0	-	
Pasteurised goat milk	1 (0)	0.00	-	2 (1)	50.00	32.26
Goat's cheese	8 (0)	0.00	-	7 (2)	28.57	30.72
TOTAL	187 (114)	60.96	-	26 (15)	57.69	-

Ct – cycle threshold

Amplification of *C. burnetii IS1111* and GAPDH, as an internal control of the extraction and amplification steps, was performed using an Adiavet Coxiella Real Time kit (Bio-X Diagnostics, Rochefort, Belgium) and a Rotor-Gene Q real-time PCR cycler (Qiagen, Hilden, Germany). The manufacturer has validated this kit for veterinary use, but it has also been applied to food products in other studies (40). The samples were treated as low bacterial burden samples when the real time PCR cycle threshold (Ct) values were ≥ 30 (17). DNA was quantified in the same way as *IS1111* was amplified. For quantification, individual standard curves were prepared for every portion of samples analysed in one real-time PCR run. The results obtained indicated that Ct values ≥ 29.71 ± 1.05 corresponded to ≤ 1.0×10^4 *C. burnetii* genome equivalents per mL.

Statistical analyses. Statistical analyses were performed using the chi-squared test available at Social Science Statistics (http://www.socscistatistics.com/).

Results

Overall, 60.56% (129/213) of the samples were positive for the presence of *C. burnetii* DNA and those were mainly pasteurised cow's milk, cheese, cottage cheese and home-style cheese, and yogurt (Table 2). The domestic-origin samples were positive in 60.96% (114/187) of cases while foreign samples were in 57.69% (15/26). However, this difference was not statistically significant ($\chi^2 = 0.01$ with Yates correction, P = 0.91). Seven out of the ten products originating from Lithuania (70%) were positive: three samples of cottage cheese, one of yogurt, one of buttermilk, and two cheese samples.

Only 26.67% (n = 4/15) of unpasteurised cow's milk samples obtained from individual farms were positive. This result was statistically significantly lower than those for the pasteurised milk produced by Latvian milk processing companies, where 76.47% of the samples (n = 13/17) were positive ($\chi^2 = 6.06$ with Yates correction, P = 0.01). Three heat-treated milk samples from a milk processing company, as well as goat's milk and goat's cheese samples from individual farms were found to be free from *C. burnetii* contamination. Other locally produced product groups had between a 53.57% (yogurts and yogurt drinks) and a 86.96% (cheese) proportion of positive samples (Table 2). Products from individual farms were positive in 12.5% of cases, and in comparison, 70.96% of samples from milk processing companies contained *C. burnetii*, and this difference was statistically significant ($\chi^2 = 35.68$ with Yates correction, P = 0.00001).

The results for products from the four most-represented milk processing companies in Latvia are given in Table 3. Statistically significant differences were obtained between companies C and A ($\chi^2 = 11.39$ with Yates correction, P = 0.0007) and C and B ($\chi^2 = 6.47$ with Yates correction, P = 0.01).

In the present study, 86.96% (n = 20/23) of cow's milk cheese samples of Latvian origin were positive: nine semi-hard ripened, four unripened/smoked, and seven cheese spread. None of the four domestic goat's milk cheese samples was positive and only two out of seven (28.57%) goat's milk cheese samples of foreign origin were.

Table 3. Results of the analysis of the pasteurised milk and milk product samples from the four largest milk processing companies in Latvia

Company	Number of samples tested / number of positive samples (%)
A	31/27 (87.10)
B	24/19 (79.17)
C	13/4 (30.77)
D	10/6 (60.00)

Table 4. Results of the analysis of the unfermented and fermented products

	Unfermented products	Fermented products (number of positive samples/ total number of samples, percentage of positive samples)		
		Without fermenting cultures	With fermenting cultures	With fermenting cultures and probiotics
Individual farms	5/18, 27.78%	0/2, 0%	1/9, 11.11%	0/6, 0%
Milk processing companies	23/35, 65.71%	10/13, 76.92%	70/99, 70.71 %	20/31, 64.52%
Total	28/53, 52.83%	10/15, 66.67%	71/108, 65.74%	20/37, 54.05%

The largest part of the positive samples were low bacterial burden samples because the real-time PCR Ct values were ≥ 30. Only 46 of the positive cow's milk products had Ct ≤ 30, mainly unpasteurised milk (2 samples), pasteurised milk (9), yogurts (4), cheese (14), cottage cheese (14) and protein drinks (2) that originated from Latvia and Lithuania, as well as one goat's cheese sample from Spain. Among the nine UHT products, seven were positive (77.78%) with Ct ≥ 30. The average Ct values and range of variation are given in Table 2.

Fermented milk products were positive in 63.13% of cases (Table 4). Of the samples with sweeteners and fruit ingredients 71.43% were positive (40/56). Products with spices and meat were less contaminated at 66.67% (7/11).

Discussion

Some studies led researchers to assume that people were infected with *C. burnetii* by consuming contaminated raw milk or home-made cheese (21, 25, 37). One study showed the presence of viable bacteria in cheese produced from unpasteurised milk (1). A research group from the USA performed an experiment in immunocompetent mice which were intragastrically inoculated by gavage with previously isolated *C. burnetii* of the three sequence types - Nine Mile (ST16), CM-SC1 (ST20), and GP-CO1 (ST8). Each strain was also directly injected into the peritoneal cavity of mice as a positive control, resulting in marked signs of infection. All strains administered directly to the stomach by oral gavage also caused infection in the experimental mice, as evidenced by the researchers detecting *C. burnetii* DNA in various tissues and elevated antibody titres (28). Additionally, some evidence of bacterial spread within the bodies of guinea pigs after administration *per os* was observed in a study in Poland (23).

The infection route from food to human in the case of Q fever has been neglected in several studies focusing on the One Health approach (29, 33). However, *C. burnetii* was considered an important foodborne pathogen in milk in earlier studies (10, 14, 22, 26). A research group from Italy concluded that the detection of *C. burnetii* should be included in the microbiological criteria for raw milk, especially when the milk is intended for direct human consumption (31). Therefore, it is important to assess milk quality at the farm level and to measure the effectiveness of milk pasteurisation methods at the dairy processing companies in Latvia and other countries, in order to ensure the protection of consumer health. Food microbiologists, in turn, should test food products for the presence and viability of *C. burnetii* bacterial cells.

Samples of Latvian origin were positive in 60.96% (114/187) of cases, while samples of foreign origin were in 57.69% (15/26) of cases. Among the 10 products of Lithuanian origin, seven samples (75%) were positive: three samples of cottage cheese, one of yogurt, one of buttermilk, and two of cheese. Such a high proportion of positive test results in food samples from Lithuania can be explained by the relatively high prevalence of infected dairy cattle herds (52.15%) in that country (34).

Only 26.67% (n = 4/15) of unpasteurised cow's milk samples obtained from individual farms were positive, compared to 76.47% (n = 13/17) of positive pasteurised cow's milk samples produced by dairy processing companies. Unpasteurised cow's milk containing *C. burnetii* DNA has to be considered an especially high-risk product, because it is the responsibility of the consumer to boil it before consumption.

Overall, the products from individual farms were positive in 12.5% of cases, compared to 70.96% positivity in samples from dairy processing companies. The proportion of positive samples depended on the company (Table 3). In previous studies from France, Spain and Hungary more frequent positive results were also obtained from industrial dairy producers than from small-scale producers (11, 17, 19). This can be explained by the smallness of the batches in which unpasteurised milk produced from a few cows by individual farms is processed and by the direct nature of the sale of that milk, which contrasts with the practice of large dairy processing companies of collecting milk from a wide area, making it possible for milk from a few infected herds to contaminate the entire production chain (11).

In the present study, 86.96% (n = 9) of Latvian cow's milk cheese samples tested positive. This percentage was significantly higher than the result of an investigation carried out in southern Italy, where only 39% of cow's milk cheese samples were positive (7). Among the product types in the current investigation, various hard, semi-hard, and smoked cheeses and cheese spread gave the highest percentages of positive tests, probably due to the relatively low temperature of pasteurisation.

None of the four domestically produced goat's cheese samples was positive and only two out of seven (28.57%) goat's cheese samples of foreign origin were. This incidence was lower than in a study from Italy, where 65.38% of industrially produced goat's cheese samples were positive (17).

The majority of the positive samples (64.34%) in this study can be treated as low bacterial burden samples, because the real time PCR Ct values were ≥30 as evaluated in a previous study (17). However, 46 (35.66%) of the positive cow's milk products had Ct ≤ 30. Taking into account the quantification results of the present study, Ct values ≥ 29.71 ± 1.05 corresponded to ≤1.0×10^4 *C. burnetii* genome equivalents per millilitre. According to a previous study, one bacterium can cause infection and five bacteria can cause the disease (4). Positive cow's milk products that had Ct ≤30 were mainly pasteurised milk, cheese and cottage cheese samples, and they originated from Latvia and Lithuania, as well as one goat's cheese sample from Spain. Among the nine UHT products, seven were positive (77.78%) with Ct ≥ 30. In general, the Ct values of the real-time PCR were comparable to those obtained in other studies. For example, the Ct values of cheese samples ranged from 26.22 to 33.50, which was similar to those in a study of unpasteurised sheep's milk cheese from Spain (1).

Paediatric infection from dairy products has been described (25); in general, children can be frequently infected with *C. burnetii* (20, 35, 36). Therefore, special attention was paid to products that could be more attractive to children in offering added chocolate, cacao, berries or other fruit. Of the sweetened samples with fruit 71.43% were positive, indicating a possible hazard.

The present study generally indicates that the incidence of *C. burnetii* DNA in milk and dairy products can be significantly higher than the incidence of infected dairy farms in a particular country. During the present investigation, 60.96% (n = 114/187) of milk and dairy product samples were found to be positive, whereas only 10.7 –13.2% of dairy cattle farms were previously identified as infected in Latvia (3). Similar results were obtained in Poland, where 31.54% of farms were infected but there was 69.16% incidence of positive tests in retail milk and dairy products (40), and in Switzerland, where the occurrence of *C. burnetii* among cheese producers exceeded the level expected from the epidemiological data on cattle herds (15).

According to the results of this investigation, we suggest that shedding of viable *C. burnetii* bacteria *via* milk should be monitored by compulsory testing, and only milk from healthy, non-shedding animals should be allowed onto the market as unpasteurised milk for direct human consumption. Furthermore, heat treatment is always recommended, as fresh milk can be a source of other pathogens that cannot be detected by standard milk testing methods. We conclude that raw milk quality and the effectiveness of milk heat treatment and pasteurisation methods at the dairy processing companies in Latvia and other countries should be carefully assessed to ensure adequate consumer health protection.

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