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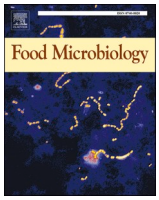

Surveillance of berries sold on the Norwegian market for parasite contamination using molecular methods

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ABSTRACT

The risk of foodborne parasite infection linked to the consumption of contaminated fresh produce has long been known. However, despite epidemiological links between the outbreaks and contaminated berries, few studies have assessed the magnitude of parasite contamination on fresh produce sold in Europe. The present study was aimed to address the knowledge gap on parasite contamination of berries sold in Norway. Samples of blueberries, strawberries, and raspberries were analysed by multiplex qPCR for detection of *Echinococcus multilocularis, Toxoplasma gondii,* and *Cyclospora cayetanensis*. In addition, a simplex qPCR method was employed for detecting contamination of the berries with *Cryptosporidium* spp. A total of 820 samples of berries, each of around 30 g (274 samples of blueberries, 276 samples of raspberries, and 270 samples of strawberries), were analysed. We found an overall occurrence of 2.9%, 6.6%, and 8.3% for *T. gondii, C. cayetanensis,* and *Cryptosporidium* spp., respectively, whereas *E. multilocularis* was not detected from any of the samples investigated. Strawberries and raspberries were most often contaminated with *Cryptosporidium* spp., whereas blueberries were contaminated mostly with *C. cayetanensis*. Detection of parasite contaminants on fresh berries indicates the need for a system to ensure the parasitological safety of fresh berries.

1. Introduction

Foodborne parasites (FBP) represent an important public health problem throughout the world, causing significant loss of disability-adjusted life years (DALYs) (Torgerson et al., 2015). People are infected from consuming food containing the infective stages of the parasites; the parasites may be contaminants of the food (which may include fresh produce), or, for some parasites, the food product may be derived from an infected animal.

For fresh produce, contamination may occur at any point in the farm-to-fork chain, warranting the application of Hazard Analysis and Critical Control Points (HACCP) (McClure et al., 2012). The potential of illness occurring due to consumption of contaminated food is particularly high if the food undergoes minimal/no processing, as is often the case with salad vegetables or fresh fruit such as berries.

Several outbreaks have been linked to the consumption of contaminated fresh produce, including berries, resulting in morbidities and hospitalisations. The case of cyclosporiasis outbreaks recorded every year in the U.S. is a typical example (https://www.cdc.gov/parasites/cyclosporiasis/outbreaks/foodborneoutbreaks.html; Ortega and Robertson, 2017).

Despite the indications of epidemiological links between the outbreaks and contaminated berries, parasites are infrequently detected in foods that are epidemiologically linked to outbreaks or cases. This often reflects the relatively prolonged periods between infection, symptoms, and diagnosis, and also the relatively short shelflife of implicated products that have frequently been consumed or discarded before infection is discovered (EFSA, 2018). Nevertheless, there have been few studies conducted to assess the magnitude of parasite contamination on fresh produce sold in the Norwegian market (Johannessen et al., 2017; Robertson and Gjerde, 2001). The lack of such studies might be partly due to the absence of reliable analytical methods for the detection of parasite contaminants. However, in the past decade, there have been substantial improvements in the development and use of molecular methods, such as quantitative polymerase chain reaction (qPCR), for the detection of different parasites from food matrices such as berries (de Souza et al., 2016; Hohweyer et al., 2016; ISO, 2016; Lalonde and Gajadhar, 2016; Murphy et al., 2018; Temesgen et al., 2019a; Temesgen

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et al., 2019b; Temesgen et al., 2021). Molecular methods have also been suggested as tools for assessing the viability of some parasite transmission stages (Rousseau et al., 2018).

The present study was therefore aimed at addressing the knowledge gap on the status of parasite contamination of berries sold in the Norwegian market. Berry production in many parts of the world has expanded considerably in recent years, reflecting increased demand and consumption in many countries, including in Europe (Tefera et al., 2018). In Norway, the relatively short growing season means that many berries are imported, with over 13,000 tonnes imported to Norway in 2020 (NFGF, 2021). Although many parasites could be transmitted to humans via contaminated berries, the present study's focus was limited to three protozoan parasites, *Toxoplasma gondii, Cyclospora cayetanensis,* and *Cryptosporidium* spp., and a helminth, *Echinococcus multilocularis*. Of these parasites, *E. multilocularis, T. gondii, and Cryptosporidium* spp. have been ranked as being among the top-5 in importance among foodborne parasites in Europe (Bouwknegt et al., 2018). More recently, a similar multi-criteria risk ranking and source attribution of food and waterborne pathogens in Norway showed that *T. gondii* and *E. multilocularis* were among the top 3, whereas *Cryptosporidium* spp. ranked 9th among the 20 pathogens considered in the risk ranking exercise (VKM, 2021). Although *C. cayetanensis* was not highly prioritized in Europe, the many foodborne outbreaks of cyclosporiasis in North America, along with the potential for its introduction in the era of globalisation of food supply, means that information on the extent of contamination of fresh produce in Europe with this parasite could be of considerable value.

2. Materials and methods

2.1. Sampling and sample size estimation

Three main types of berries were considered in the present study: blueberries, strawberries, and raspberries. The berries used in the survey were provided by both Coop Norge and Bama-Gruppen AS, which both import fresh produce, including berries, and distribute these, along with Norwegian-grown produce, to the market. The sampling period was between August 2019 and November 2020.

The minimum number of samples required for the survey was calculated using the formula presented in equation (1). The estimated proportion of contaminated berries was considered 20% for *E. multilocularis* (Lass et al., 2015) and 9.7% for *T. gondii* (Lass et al., 2012), the desired level of precision at 3%, and assuming a 95% confidence level, the minimum number of samples was estimated at 683.

$$n = z^2 * \frac{p(1-p)}{e^2} \quad (1)$$

where:

- n is the minimum number of samples to be investigated,
- z is the Z score related to the confidence level,

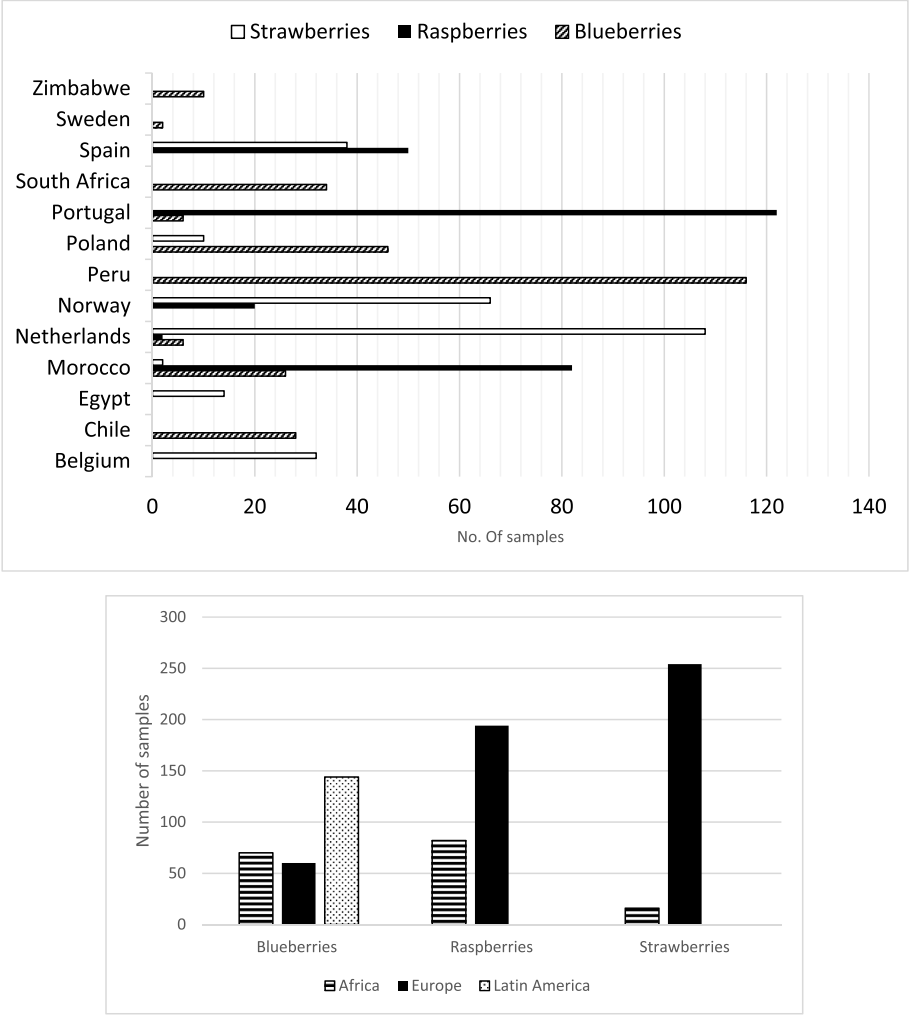


Fig. 1. The distribution of sources of berries, country-wise (top graph) and continent-wise (bottom graph), analysed by molecular methods for contamination with parasites.

- p is the estimated proportion, and
- e is the desired precision of the estimate.

A total of 820 samples of berries, which included 274 samples of blueberries, 276 samples of raspberries, and 270 samples of strawberries, were analysed for parasite contamination. Each sample was composed of approximately 30 g of berries that was made up of individual berries taken from each of the punnets provided in a crate by the distributor, until the required weight of berries was reached. Most of the samples were from imported berries, whereas 86 samples (66 strawberries and 20 raspberries) were berries produced in Norway. The import countries were pragmatically prioritized based on the known geographical distribution of the parasites under investigation. Countries such as Peru, Morocco, Chile, and Poland are among the priority import countries (Fig. 1).

2.2. Sample processing

The berries' surfaces were subjected to a washing procedure in Alconox solution (Sigma Aldrich, Norway) in order to remove any contaminant parasite stages. Briefly, 200 ml of 0.1% Alconox was added to a plastic box containing about 30 g of berries. The box was closed and put on a shaker (VibraMax) with a setting of 600 rpm for blueberries and strawberries, but at a lower setting of 300 rpm for raspberries due to their greater fragility, for 10 min. The elution step was followed by a concentration step in which the eluate (200 ml) was concentrated down to a volume appropriate for the pre-selected detection method. Briefly, the eluted solution was carefully transferred to four 50 ml conical tubes and then centrifuged at 1690×*g* for 10 min. The supernatants were removed by vacuum suction from each tube, keeping 10 ml of the pellet and the liquid above the pellet, and these were then combined into a single tube. Although the tubes used were disposable, the boxes were reused. They were first rinsed by hand, then washed on a hot cycle of a conventional dishwasher. Cross contamination between samples was considered in the results, but not observed.

This tube was then centrifuged again at 3803×*g* for 10 min. The supernatant was removed, leaving about 1.5 ml of sediment, which was then transferred to a microcentrifuge tube after vortex mixing. The microcentrifuge tube was then spun at 13,000×*g* for 5 min. The supernatant was removed and the remaining 250 μL sediment was used for DNA extraction as described in section 2.3.

2.3. DNA extraction

The concentrated sediment was subjected to DNA extraction using DNeasy PowerSoil Kit (Qiagen, Norway). Briefly, 250 μL of the sediment was mixed with 60 μL of the Solution C1 in a PowerBead tube provided with the kit (already containing buffer and beads) and were subjected to bead-beating to facilitate the release of DNA. Bead-beating was performed using FastPrep-24 5G™ High Speed Homogeniser (MP Biomedicals, Illkirch Cedex, France) in two cycles of 4 m/s for 60 s with 45 s pause between the cycles. The lysate was then centrifuged at 10,000×*g* for 1 min at room temperature, and 500 μL of the supernatant used for the subsequent step in the protocol. The DNA was eluted in 50 μL of Solution C6. Samples were stored at −20 °C until further analysis.

2.4. Real-time PCR (qPCR)

A previously published multiplex qPCR protocol was employed for the detection of *E. multilocularis, T. gondii,* and *C. cayetanensis* (Temesgen et al., 2019a). In addition, *Cryptosporidium* spp. was detected using a protocol published previously ((UKWIR, 2020); Elwin et al., personal communication - paper in preparation). The list of oligonucleotides used in the qPCR protocols is given in Table 1. The qPCR was performed in Stratagene Mx3005P of Agilent Technologies (Matriks As, Norway).

Table 1
List of oligonucleotides used for the detection of *E. multilocularis, T. gondii, C. cayetanensis,* and *Cryptosporidium* spp.

Oligonucleotides	Sequence (5′-3′)	Reference
Oligonucleotides used for qPCR		
C. cayetanensis		Temesgen et al. (2019)
CyITS1_TT-F	ATGTTTTAGCATGTGGTGTGGC	
CyITS1_TT-R	GCAGCAACAACAACTCCTCATC	
CyITS1_TT-P	HEX-TACATACCCGTCCCAACCCTCGA-MGBEQ	
T. gondii		
Tox-9F	AGGAGAGATATCAGGACTGTAG	
Tox-11R	GCGTCGTCTC GTCTAGATCG	
Tox-TP1	Cy5-CCGGCTTGGCTGCTTTTCCT-MGBEQ	
E. multilocularis		
EmMGB_F	GTGCTGCTYATAAGAGTTTTTG	
EmMGB_R	CTATTAAGTCCTAAACAATACCATA	
EmMGB_P	FAM-ACAACAATATTCCTATCAATGT-MGBEQ	
Cryptosporidium spp.		
JF1	AAGCTCGTAGTTggatTTCTG	UKWIR (2020); Elwin et al, personal communication - paper in preparation
JF2	AAGCTCGTAGTTaatcTTCTG	
JR	TAAGGTGCTGAAGGAGTAAGG	
JT2	Cy5-TCAGATACCGTCGTAGTCT-MGBEQ	
Oligonucleotides used for MLST of *C. cayetanensis*		
CyC21_F1	TAGTGGCGACTGCGACATG	Guo et al. (2016)
CyC21_R1	GCACCTTGCTGATGAGGCA	
CyC21_F2	CTAAGGCTGTCTTGAGCGG	
CyC21_R2	CGCCCACATGCTTCGTATAC	
CYC13_F1	TTGGAGCAGGACGAGTTTCG	
CYC13_R1	ATGGAAGCGGCTATGAAATTGG	
CYC13_F2	CCTCGGAGTCCTCTGAGTG	
CYC13_R2	AGCCGTCGCAGTGTGTAGCA	

2.4.1. Multiplex qPCR

The multiplex qPCR was performed in a 20 μL reaction volume that included 10 μL of KiCqStart® Probe qPCR ReadyMix™, low ROX™ (Sigma-Aldrich, Norway), 2 μL of template, 0.5 μM of primers of *C. cayetanensis* and *T. gondii*, 0.4 μM of primers of *E. multilocularis*, 150 nM, 250 nM, and 130 nM of probes targeting *C. cayetanensis, T. gondii,* and *E. multilocularis*, respectively. The thermal cycling condition was as follows: initial denaturation at 95 °C for 3 min followed by 45 cycles of denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 30 s. Nuclease-free water was used as no template control (NTC) and genomic DNA extracted from *T. gondii* and C. *cayetanensis,* as well as a plasmid of *E. multilocularis,* were used as positive controls in each run.

2.4.2. qPCR for detection of Cryptosporidium spp.

The qPCR protocol for the detection of *Cryptosporidium* spp. was performed in a 25 μL reaction volume that consisted 12.5 μL of KiCqStart® Probe qPCR ReadyMix™, low ROX™ (Sigma-Aldrich, Oslo, Norway), 5 μL of template, 0.6 μM of the primers, and 80 nM of the probe. The forward primer was prepared as a 1:1 mixture of JF1 and JF2. In each qPCR run, genomic DNA extracted from *C. parvum* was included to serve as a positive control; nuclease-free water was used as NTC.

2.5. Conventional PCR and sanger sequencing

Of the samples that tested positive for *Cyclospora* with the multiplex qPCR protocol, 16 (8 raspberries, 7 blueberries, and 1 strawberry) were selected for further analysis (on the basis of low Ct value), by conventional PCR (multilocus sequence typing; MLST) and Sanger sequencing as described in section 2.5.1. In addition, 8 strawberry samples with *Cryptosporidium*-positive qPCR results were selected and sent for Sanger sequencing.

2.5.1. Multilocus sequence typing (MLST)

Berry samples positive for *Cyclospora* qPCR were selected and subjected to MLST targeting CYC13 and CYC21 targets according to a previously described protocol (Guo et al., 2016) with slight modifications. Briefly, nested PCR involving 250 nM of each primer, 12.5 μL of DreamTaq Green PCR Master Mix (2X), and 2 μL of template in a reaction volume of 25 μL was conducted. The PCR was setup with an initial denaturation of 95 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 15 s, annealing at 55 °C (CYC21) or 58 °C (CYC13) and for 45 s and 72 °C for 1 min.

2.6. Statistical analyses

Chi-square and Fisher's exact tests were used to assess the statistical significance of differences in the frequencies of parasite contamination among the different berry types. Differences were considered significant at $p \leq 0.05$.

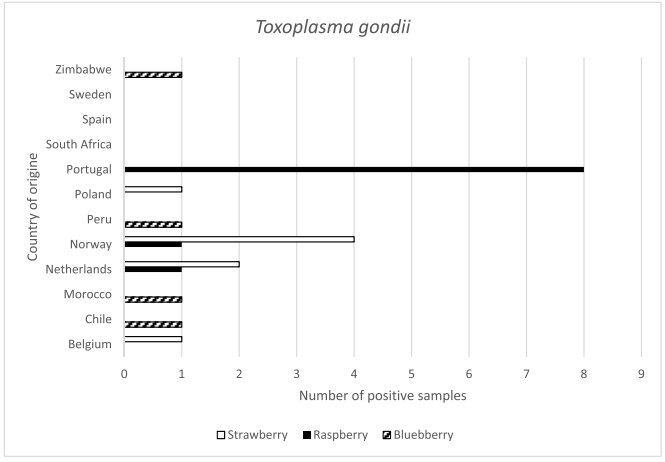
3. Results

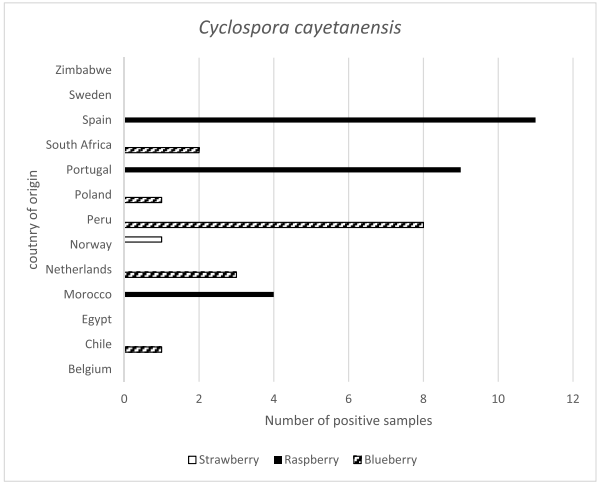
Importantly, *E. multilocularis* was not detected in any of the samples analysed in the present study. The overall occurrence of *Toxoplasma gondii, C. cayetanensis*, and *Cryptosporidium* spp. was 2.9% (24/820), 6.6% (52/820), and 8.3% (68/820), respectively (Table 2). The findings of the present study showed that raspberries were the most contaminated berries followed by strawberries and blueberries. *Cryptosporidium* spp. was detected most often from strawberries followed by raspberries, and blueberries were contaminated less often; this difference was statistically significant. For both *Toxoplasma* and *Cyclospora*, raspberries were the berry type most frequently found to be contaminated, although there were no significant differences for either parasite.

Fig. 2 shows the distribution of country of origin for the berry samples found to be contaminated with the parasites. The sources of berries contaminated with *Toxoplasma* include Chile, Poland, Norway, and Zimbabwe. Both *Toxoplasma* and *Cyclospora* contamination were detected on berries imported from Portugal, Morocco, Belgium, and the Netherlands.

Of the 16 samples submitted for Sanger sequencing for the *Cyclospora* MSLT, only 3 samples had a BLAST match with high percentage identity (99%, 98%, and 88%) to sequence deposits in GenBank (KP723495.1, KY770764.1, KX273389.1, KP723508.1). The samples included two from blueberry and one from raspberry samples that had positive PCR results. All 3 samples showed alignment with GenBank sequences similar to *Cyclospora cayetanensis* for the CYC13 target, whereas one of the blueberry samples showed alignments for both the CYC13 and CYC21 targets.

Of the 8 strawberry samples with *Cryptosporidium*-positive qPCR tests that were submitted for Sanger sequencing, four of the sequences obtained had BLAST matches with percentage identity ranging from 84.3% to 97.6% with GenBank sequences for *Cryptosporidium*: EF613339.1 (non-specified species), KT027481.1 (skunk genotype), MN577592.1 (*C. parvum*), MN577592.1 (*C. parvum*). These samples originated in the Netherlands (sample with similarity to EF613339.1) and Norway (all other samples).





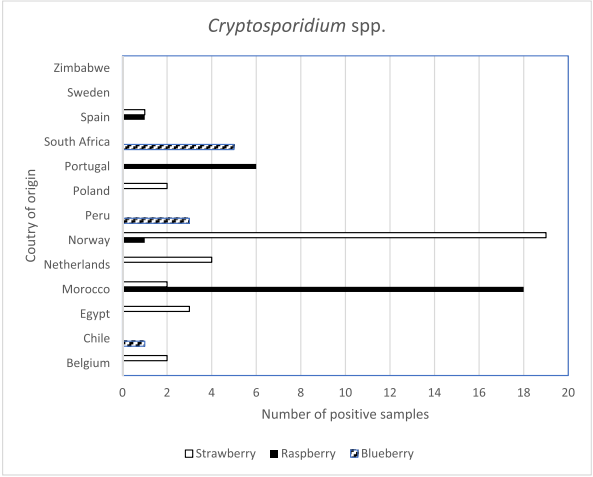


Fig. 2. Countries of origin of berries contaminated with parasites.

Table 2
The frequency distribution of parasite contaminants on berries.

Berry type	No. analysed (% of total)	Parasites detected; number positive (%)			
		Echinococcus multilocularis	*Toxoplasma gondii*	*Cyclospora cayetanensis*	*Cryptosporidium* spp.
Blueberries	274 (33.4)	0	4 (1.5)	15 (5.5)	9 (3.3)
Raspberries	276 (33.7)	0	10 (3.6)	24 (8.7)	26 (9.4)
Strawberries	270 (32.9)	0	8 (2.9)	13 (4.8)	33 (12.2)
Total	820	0	24 (2.9)	52 (6.6)	68 (8.3)

4. Discussion

In the present study, the analysis of berries for parasite contamination using molecular methods showed variable frequencies of contamination with the parasites under investigation. Notably, *E. multilocularis* was not detected from any of the berry samples analysed. Although an apparent absence of contamination with *E. multilocularis* might not be unexpected, our results contrast with those from a study in Poland in which 20% of raspberry samples were reported to be contaminated with *E. multilocularis* (Lass et al., 2015). Although we did not analyse Polish raspberries in our study, we analysed 10 strawberry samples and 46 blueberry samples from Poland, which may be speculated to be more likely than raspberries to be contaminated with this parasite, due to the shorter distance of those berries from the ground during cultivation, and thus the greater likelihood of contamination. Whether the Polish results accurately reflect actual levels of contamination of raspberries has been debated in the literature, including regarding whether the berry height predisposes to contamination and the potential role of flies (Robertson et al., 2016; Torgerson, 2016). Other European countries considered endemic for alveolar echinococcosis, such as France, Austria, Germany, and Switzerland, were not among the exporters of berries to the Norwegian market and therefore were not included in the present study. Interestingly a recent study from Italy detected *E. multilocularis* contamination of one sample of ready-to-eat salad (1.4% of samples) using the same multiplex qPCR protocol used in our study (Barlaam et al., 2021).

Regarding the other parasites in focus, *T. gondii, C. cayetanensis,* and *Cryptosporidium* spp., various levels of contamination were detected, ranging from just under 3% with *Toxoplasma* to just over 8% with *Cryptosporidium*. The detection of parasite contaminants from berries' surfaces has significant public health implications; however, it should be noted that only DNA was detected, so we cannot be certain that the intact, infective stages were present, and, if they were, we have no information on their viability. Nevertheless, based on information in the literature it is clear that infections can occur with these parasites due to contaminated fresh produce, and the detection of contamination here should give rise to some degree of concern among the Norwegian food-safety authorities. For *Toxoplasma*, an outbreak in Brazil was associated with contaminated salad (Dubey, 2021). Multiple outbreaks of cyclosporiasis in North America have been linked to consumption of contaminated produce, including berries (Almeria et al., 2019). In addition, various outbreaks of cryptosporidiosis have been associated with produce, although not specifically berries, including in Scandinavia (Robertson and Chalmers, 2013). In particular, one small outbreak of cryptosporidiosis in Norway was linked to consumption of contaminated apple juice (Robertson et al., 2018). Although our data, which indicates relatively high occurrence of contamination, may be alarming, consumer-washing of berries before consumption has been shown to be effective at removing a considerable proportion of parasite contaminants (Temesgen et al., 2021).

Previous surveillance in Norway of fresh produce, including berries, for contamination with parasites focussed on *Cryptosporidium*, *Giardia, Cyclospora,* and *Ascaris* (Robertson and Gjerde, 2001). Neither *Ascaris* nor *Cyclospora* were detected among the samples examined (85 samples examined for *Cyclospora*, and 300 for *Ascaris*), but *Cryptosporidium* and *Giardia* were found in some of the 475 samples examined for these parasites (4% and 2% positive, respectively). Relatively few berry samples were examined, and two samples of fresh strawberries were found to be contaminated with *Giardia* cysts, but no *Cryptosporidium* was detected among the 72 berry samples examined. This relative apparent lack of contamination from these earlier studies could reflect the lower sensitivity of microscopy compared with the qPCR method used in our study. However, it could also indicate that the contamination is largely with DNA, rather than the transmission stages that are identified by microscopy-based methods. These could also be the possible reasons for why *Cyclospora* was not detected at all in the earlier Norwegian survey – although this may also reflect that the potential for contamination with *Cyclospora* has increased in the two decades since the previous survey was conducted. To the best of our knowledge, ours is the first study to use molecular methods to investigate contamination of berries in the Norwegian market with parasites of public health relevance. The sensitivity of the multiplex qPCR used in the present survey was estimated to be 10 oocysts of *Toxoplasma* and *Cyclospora* per 30 g of berries. However, limitations pertaining to the amount of berries used for analysis and the multistep procedure in the detection of parasites means that there could be underestimation of the contamination.

Two other studies that used the same multiplex assay used in our study have directly comparable results, although the sampling regimes varied somewhat. These studies involved analysis of strawberries grown in Colombia (Pineda et al., 2020) and ready-to-eat salads and berries sold in Italy (both Italian and imported produce; Barlaam et al., 2021). Whereas the *Toxoplasma* results from Colombian strawberries were higher than our results at 5% (6/120; Pineda et al., 2020), none of the salad samples from the Italian market were positive for *Toxoplasma* (Barlaam et al., 2021). Our data appear to have been elevated by a relatively high occurrence of *Toxoplasma*-positive raspberry samples imported from Portugal, which may indicate a necessity for investigating the farm-to-shipping chain for this produce. Similarly, for *Cyclospora* the occurrence of contamination found in our study was 6.6%, whereas in the studies from Colombia (Pineda et al., 2020) and from Italy (Barlaam et al., 2021), only 1 sample was found to be positive in each study. It is not clear why the occurrence of *Cyclospora*-positive samples was much higher in Norway, but may reflect the greater diversity of samples in our study in terms of origins of the produce.

Regarding *Cryptosporidium*, it is worth noting that although positive samples were found in produce from 11 countries, the greatest frequency was actually among Norwegian strawberry samples, perhaps reflecting the known propensity for the transmission stages of this parasite to survive best in cool, damp conditions (Utaaker et al., 2017; Medema et al., 2005; Alum et al., 2014). The molecular investigations of the *Cryptosporidium* species found in our study provided supporting evidence for the presence of this parasite, with both *C. parvum* and skunk genotype indicated. Although *C. parvum* infects humans and a range of other animals, the host range for skunk genotype has yet to be established although it has been identified in skunks, racoons, and squirrels (Xiao et al., 2008). The strawberries contaminated with these species/genotypes of *Cryptosporidium* originated from Norway. Although *C. parvum* is widespread in Norway and has been associated with several outbreaks (e.g., Robertson et al., 2019; Lange et al., 2014) to date, skunk genotype has never been reported here. However, unlike in other European countries, molecular investigations of infecting species in cryptosporidiosis cases is only sometimes conducted in Norway, such as in outbreak investigations (van der Giessen et al., 2021), and this zoonotic genotype has been identified in cases elsewhere in Europe (e.g., UK; Elwin et al., 2012) and is considered to be commonly identified in surface water (Yan et al., 2017).

5. Conclusion

Our results indicate that parasite contaminants relevant to public health occur commonly on fresh berries sold on the Norwegian market, although we found no evidence of contamination with *E. multilocularis*. Implementation of HACCP for certain produce chains may be indicated, and periodic surveillance of berries for parasite contamination may be of value. However, although there is a public health indication, encouraging consumers to wash berries before consumption should reduce the risk of infection.

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Conflict of interest and authorship conformation form

Please check the following as appropriate:

X All authors have participated in (a) conception and design, or analysis and interpretation of the data; (b) drafting the article or revising it critically for important intellectual content; and (c) approval of the final version.
X This manuscript has not been submitted to, nor is under review at, another journal or other publishing venue.
o The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript
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None of these companies had any influence on the work described here, but two of the companies provided the berries for analysis. These berries would otherwise have been sold on the open market.

The salary of two authors, TTT and VMA, was provided directly from the project funding.

The companies providing additional funding are listed under "funding" within the manuscript.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

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