



Adaptation and validation of a droplet digital PCR assay for the detection of *Cryptosporidium* spp. and *Giardia duodenalis* in buffalo milk

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ABSTRACT

Cryptosporidium spp. and *Giardia duodenalis* are major protozoan enteropathogens of public health and food-safety concern. Their detection in dairy matrices remains challenging due to the lack of standardized analytical protocols and the limited sensitivity of conventional diagnostic methods. In this study, we developed and validated a droplet digital PCR (ddPCR) assay for the detection of *Cryptosporidium* spp. and *G. duodenalis* in buffalo milk. The method showed satisfactory recovery efficiencies (61% for *G. duodenalis* and 55% for *Cryptosporidium* spp.) and excellent analytical performance, including a limit of detection of 1 gc/μL, 100% specificity, and strong intra- and inter-day repeatability. The operator-independent robustness of the dd-PCR platform further supports its suitability for routine diagnostics in complex dairy matrices. Application of the validated assay to 44 buffalo milk samples collected from two dairy farms in the Campania Region (southern Italy) revealed prevalences of 31.8% for *G. duodenalis* and 18.2% for *Cryptosporidium* spp. A higher number of positive samples was observed in May, suggesting a possible seasonal influence; however, this indication is preliminary and cannot be interpreted as a confirmed seasonal pattern. Overall, the findings demonstrate that the proposed dd-PCR assay is a highly sensitive and reliable tool for protozoan surveillance in buffalo milk and highlight the need for strengthened monitoring during high risk seasonal periods.

1. Introduction

Cryptosporidium spp. and *Giardia duodenalis* are protozoan parasites that infect the intestinal epithelium of humans and animals, causing diarrhea, malabsorption, and impaired growth (Wróblewska et al., 2025). Their global relevance is well established. Data from the Global Enteric Multicenter Study (GEMS), which monitored more than 7800 children across South Asia and Sub-Saharan Africa, demonstrated a strong association between infection, whether symptomatic or asymptomatic, and subsequent growth faltering in children under five years of age (Das et al., 2023). The World Health Organization recognized these protozoa as major contributors to childhood morbidity as early as 1987

(WHO Expert Committee, 1987), and a recent FAO/WHO multicriteria ranking reaffirmed their status as priority foodborne parasites (FAO/WHO, 2024).

Ruminants, including water buffalo (*Bubalus bubalis*), cattle (*Bos taurus*), and zebu (*Bos taurus indicus*), are important reservoirs of *Cryptosporidium* spp. and *G. duodenalis*, contributing to zoonotic transmission through environmental dissemination of (oo)cysts (Sahin et al., 2025; Hatam-Nahavandi et al., 2019). In buffalo farming, infections are frequently subclinical in adults but may cause severe disease in neonates and immunocompromised animals, leading to substantial economic losses due to reduced productivity, treatment costs, and high within-herd prevalence (De Aquino et al., 2020).

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In Italy, buffalo milk is primarily used to produce "Mozzarella di Bufala Campana," a PDO-protected cheese processed at 85–95 °C, temperatures capable of inactivating most parasites (Rinaldi et al., 2007; Rutigliano et al., 2025; Fenga et al., 2025; Cacciò et al., 2007). Despite this intrinsic safety margin, European food-hygiene legislation (Reg. EC 852/2004; 853/2004) requires validated processing steps and strict hygiene measures to ensure microbiological safety throughout the production chain. The absence of specific regulatory frameworks and standardized detection methods for *Cryptosporidium* spp. and *Giardia duodenalis* in food matrices increases the risk of underestimating food-borne transmission. Current diagnostic approaches include microscopy, immunological assays, and molecular techniques, each with inherent limitations. Microscopy is cost-effective but requires skilled personnel and often lacks sensitivity (Elmahallawy et al., 2023). Immunological assays enable large-scale screening but do not allow species- or genotype-level differentiation (De Aquino et al., 2020). Molecular methods, including PCR-based genotyping, Sanger sequencing, and probe-based qPCR, offer higher analytical accuracy by targeting multi-copy genomic regions such as small subunit ribosomal RNA (SSU rRNA) and internal transcribed spacer (ITS) (Elmahallawy et al., 2023). SSU rRNA is a multicopy genomic region, which enhances analytical sensitivity and influences quantitative interpretation. Accurate quantification of protozoan contamination in food products is essential for risk assessment, surveillance, and the development of targeted mitigation strategies (Cazeaux et al., 2022). To address these diagnostic gaps, we developed and validated a droplet digital RT-PCR (ddPCR) assay for the detection of *Cryptosporidium* spp. and *Giardia duodenalis* in buffalo milk. Furthermore, we applied the validated method to assess the seasonal prevalence of these protozoa in dairy buffalo farms in the Campania Region, providing new insights into contamination dynamics in this high-value dairy sector. To our knowledge, no ddPCR assay has been previously adapted or validated for the detection of *Cryptosporidium* spp. and *Giardia duodenalis* in buffalo milk, nor applied to field samples from dairy buffalo farms. The present study provides the first ISO 16140-compliant validation of a ddPCR method for protozoan detection in this complex matrix and offers new epidemiological insights into contamination dynamics in buffalo milk.

2. Materials and methods

2.1. Study area and sampling

The study was conducted in the Campania Region (southern Italy), characterized by a Mediterranean temperate climate with increasingly continental features in inland and mountainous areas. Milk samples were collected between August 2023 and May 2024 from 44 dairy buffaloes reared on two intensive farms located in the provinces of Salerno and Caserta (Table 1). Animals were clinically healthy, aged 3–9 years, and produced milk with normal physical characteristics. For each animal, 50 mL of individual milk and a bulk sample were collected during evening milking under sterile hygienic conditions. Samples were immediately transported to the laboratory in refrigerated containers at 4 °C and stored at –20 °C until analysis. All samples were analyzed using the ddPCR assay described in Section 2.3. Each analytical determination was performed in triplicate.

Table 1
Collection sites in the campania region.

Sample	Sampling Period	Sampling Site
1-13	02/08/23	CASERTA
14-28	14/12/23	SALERNO
29-44	27/05/24	SALERNO

2.2. DNA extraction

For each sample, 5 mL of buffalo milk was centrifuged at 10,000 × g for 1 h at 4 °C. After centrifugation, the fat layer and supernatant were removed, and the pellet was washed with phosphate-buffered saline (PBS). Total DNA was extracted using the QIAamp DNA Mini Kit (Qia-gen, Hilden, Germany) according to the manufacturer's instructions. DNA concentration and purity were assessed using a BioPhotometer Plus spectrophotometer (Eppendorf AG, Hamburg, Germany).

2.3. Droplet digital RT-PCR (dd RT-PCR) analysis

The ddPCR assay targeted the SSU rRNA gene for both *Cryptosporidium* spp. and *Giardia duodenalis*, using primers and probes previously described by D'Auria et al. (2025). Droplet digital PCR was performed using the QX200 system (Bio-Rad, Hercules, CA, USA). All ddPCR reactions were performed in singleplex format, with *Cryptosporidium* spp. and *Giardia duodenalis* amplified in separate wells. Each 20 µL reaction contained 10 µL of ddPCR Supermix for Probes (Bio-Rad, Hercules, CA, USA), forward and reverse primers, the corresponding probe, 35–50 ng of template DNA, and nuclease-free water (Table 2).

Amplification was carried out on a CFX96 Deep Well system (Bio-Rad, Hercules, CA, USA) under the following cycling conditions: enzyme activation at 95 °C for 10 min; 45 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 45 s; and a final extension at 98 °C for 10 min. Droplets were read using the QX200 Droplet Reader (Bio-Rad, Hercules, CA, USA).

Data were analyzed with QuantaSoft v1.7 software (Bio-Rad, Hercules, CA, USA) to obtain absolute quantification (gc/µL) based on Poisson statistics.

Unless otherwise specified, all concentrations reported in this study refer to gene copies per microlitre of ddPCR reaction (gc/µL reaction).

2.4. Method validation

Reference strains of *Cryptosporidium* spp. (MBC126-R, 2 × 10⁴ gc/µL), and *Giardia duodenalis* (MBC119-R, 2 × 10⁴ gc/µL) were diluted to obtain working concentrations of 100, 10, and 1 gc/µL. Parasite-free buffalo milk was inoculated with each concentration to generate validation samples; sterile water served as the negative control. Specificity was assessed using parasite-free buffalo milk and a panel of non-target organisms relevant to dairy environments. Non-target protozoan DNA included *Toxoplasma gondii*, *Neospora caninum*, and *Eimeria* spp., obtained as certified reference genomic DNA from commercial suppliers (Microbiologics®, USA). Non-target bacterial DNA (*Escherichia coli*, *Staphylococcus aureus*, and *Listeria monocytogenes*) was extracted from previously characterized laboratory isolates routinely used for quality-control procedures in our institution.

2.4.1. Sensitivity

Sensitivity was evaluated by determining the limit of detection at 95% probability (LOD₉₅) using ten independent replicates per concentration. DNA concentration was determined by a Biophotometer (Eppendorf, Hamburg, Germany), and samples were diluted to be analyzed by ddPCR at three concentration levels: 100 gc/µL, 10 gc/µL, and 1 gc/µL gc/µL, to evaluate the LOD₉₅. For each concentration level (100, 10, and 1 gc/µL), ten independent replicates were analyzed. The LOD₉₅ was estimated using a probit regression model based on the proportion of positive results obtained at each concentration level, following ISO 16140- 2 guidelines. Concentrations below 1 gc/µL were not tested; therefore, the LOD₉₅ can only be defined within the tested range and cannot be reported as <1 gc/µL.

In accordance with ISO 16140 (ISO 16140-2:2016), the specificity value reflects only the organisms and matrices included in the validation panel and must be interpreted within these limits (Jones & Marengo, 2016).

Table 2Primers and probes used to detect *Cryptosporidium* spp. and *Giardia duodenalis*.

Primer Name	Sequence 5' - 3'	Concentrations	Source
Crypto probe	FAM—TGCCATACATTGTTGCTGACAAATTGAAT—BHQ1	240 nM	Mazzara et al. (2023)
COWP For	CAAATTGATACGTTTGTCTTCTG	600 nM	Haque et al. (2007)
COWP Rev	GGCATGTGCGATTCTAATTCAGCT	600 nM	
GD 105 probe	FAM—CCCGCGGCGGTCCCTGCTAG—BHQ1	90 nM	
GD 80 For	GACGGCTCAGGACAACGGTT	360 nM	
GD 127 Rev	TTGCCAGCGGTGTCCG	360 nM	

BHQ: Black Hole Quencher.

2.4.2. Repeatability and reproducibility

Extracted DNA was stored at -20°C in AE buffer (Qiagen) between analytical runs. To avoid degradation associated with repeated freeze–thaw cycles, aliquots were prepared immediately after extraction, and each aliquot was thawed only once for its corresponding analytical day. Intraday repeatability was assessed by analyzing ten replicates per concentration on the same day. Interday reproducibility was evaluated by repeating the same analytical scheme on two separate days. Robustness was determined by comparing assay performance between different operators using identical experimental conditions.

2.4.3. Recovery

Buffalo milk samples were artificially contaminated with certified reference DNA of *Giardia duodenalis* and *Cryptosporidium* spp. Defined aliquots corresponding to approximately 1×10^3 and 1×10^4 gene copies per sample were spiked into 10 mL of raw milk to simulate low and moderate contamination levels. Samples were homogenized and processed immediately. DNA extraction was performed using the same protocol described above. DNA was eluted in 100 μL of buffer and stored at -20°C until analysis. Quantification of recovered DNA was performed using dd-PCR, and recovery efficiency was calculated by comparing measured and theoretical gene copy numbers. This approach quantifies losses attributable to DNA extraction and provides a general indication of matrix-associated inhibition; however, it does not allow discrimination between extraction inefficiencies and inhibition occurring during ddPCR amplification. A complete inhibition assessment would require an additional experiment in which reference DNA is added post-extraction to the purified matrix. This step was not included in the present study and represents an important methodological limitation.

2.5. Statistical analyses

Prevalence was calculated as the proportion of positive samples over the total number of samples analyzed, expressed as a percentage. Exact 95% confidence intervals (CI) were computed using the Clopper–Pearson method, which is appropriate for binomial data and small sample sizes.

$$\text{Prevalence} = \frac{\text{Number of positive samples}}{\text{Total samples}} \times 100$$

The precision of the ddPCR assay was evaluated by calculating the coefficient of variation (CV%) for each concentration level. CV% values were obtained as the ratio between the standard deviation and the mean gene-copy number, multiplied by 100. This metric was used to assess both intraday repeatability and interday reproducibility.

Confidence intervals (95% CI) were computed using the Clopper–Pearson method.

Coefficients of variation (CV%) were calculated as:

$$\text{CV\%} = \frac{\text{Standard deviation}}{\text{the mean value for each level}} \times 100$$

Associations between sampling period and protozoan positivity were evaluated using chi-square or Fisher's exact tests, depending on expected

frequencies. Odds ratios (ORs) and corresponding p-values were calculated to quantify the strength of association. Statistical significance was set at $p < 0.05$. All analyses were performed using IBM SPSS Statistics v29 (IBM Analytics, Armonk, NY, USA).

3. Results

3.1. Extraction performance

The extraction protocol demonstrated consistent and satisfactory performance across all tested samples. At the 100 gc level, recovery rates were 61% for *Giardia duodenalis* and 55% for *Cryptosporidium* spp., confirming the suitability of the extraction workflow for complex, fat-rich matrices such as buffalo milk. To provide measurable evidence of extraction performance, DNA yield and purity (A260/280) were quantified for all samples, and summary values are reported in Table 3. Extracted DNA was aliquoted immediately after extraction and stored at -20°C in AE buffer (Qiagen). Each aliquot was thawed only once for its corresponding analytical day, preventing freeze thaw associated degradation.

3.2. Recovery

dd-PCR enabled the absolute quantification of *Giardia duodenalis* and *Cryptosporidium* spp. DNA was found in all spiked buffalo milk samples. Measured DNA concentrations were compared with the theoretical gene copy numbers added to each sample to estimate extraction efficiency. Recovery rates ranged: 50–80% for *Giardia duodenalis* and 40–70% for *Cryptosporidium* spp. The method showed good repeatability, with coefficients of variation below 15% across replicates. No amplification was observed in negative controls, confirming the absence of contamination.

3.3. Analytical sensitivity

The dd-PCR assay exhibited high analytical sensitivity, consistently detecting target DNA at concentrations as low as 1 gc/ μL . Following Srisutham et al. (2017), samples were classified as positive only when at least two positive droplets were present. The specificity panel included DNA from *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Toxoplasma gondii*, *Neospora caninum*, and *Eimeria* spp., all commonly encountered in dairy environments. No amplification was observed for any non-target organism. Across the full concentration range tested (1–100 gc/ μL), all replicates yielded positive amplification for both protozoa, indicating that the LOD₉₅ lies below 1 gc/ μL . The assay

Table 3

DNA yield and purity (A260/280) of extracted buffalo-milk samples.

Sample type	n	Mean DNA yield (ng/ μL)	SD	Mean A260/280	SD
Individual milk samples	44	12.8	3.1	1.78	0.06
Bulk tank samples	2	14.2	2.4	1.80	0.04
Negative controls	5	<1.0	—	1.00	—

showed 100% analytical sensitivity (detection of all replicates at tested concentrations) and specificity (absence of amplification in negative controls and non-target DNA).

Precision analysis revealed increasing CV% values at lower concentrations, as expected for digital PCR platforms.

- *Giardia duodenalis*: 1.63% (100 gc/μL), 4.28% (10 gc/μL), 17.5% (1 gc/μL)
- *Cryptosporidium* spp.: 1.76% (100 gc/μL), 4.55% (10 gc/μL), 14.75% (1 gc/μL)

No false-positive droplets were detected in negative controls (Table 4).

3.4. Analytical precision

Intraday results (Table 5) confirmed high repeatability, particularly at 100 and 10 gc/μL, where CV values remained well below the commonly accepted threshold for dd-PCR precision (<5%). Variability increased at 1 gc/μL, consistent with assays operating near the limit of detection.

Interday results (Table 6) demonstrated excellent reproducibility across three independent days. Even at 1 gc/μL, the assay maintained coherent performance, although variability increased as expected at low target concentrations.

3.5. Buffalo milk analyses

Screening of 44 buffalo milk samples collected between August 2023 and May 2024 revealed 31.8% positivity for *Giardia duodenalis* (95% CI: 18.1–48.1%) and 18.2% positivity for *Cryptosporidium* spp. (95% CI: 8.2–32.7%). Positive samples were distributed across all sampling periods, with a marked increase in detections in May, suggesting a potential seasonal contamination trend (Table 7).

Gene-copy concentrations in positive samples ranged from 0.19 to 3.7 gc/μL for *Giardia duodenalis* and 0.7 to 6.9 gc/μL for *Cryptosporidium* spp. These findings reflect the high analytical sensitivity of dd-PCR and highlight the presence of low-level protozoan contamination in buffalo milk from the studied farms.

4. Discussion

This study reports the adaptation and validation of a dd-PCR assay for the detection of *Cryptosporidium* spp. and *Giardia duodenalis* in buffalo milk, addressing a critical diagnostic gap in food-safety surveillance.

Compared with conventional PCR and qPCR, ddPCR offers improved analytical sensitivity, absolute quantification without calibration curves, and greater resilience to matrix-associated inhibitors. These advantages are particularly relevant for fat-rich matrices such as buffalo milk, where qPCR inhibition is frequently reported (Guri et al., 2024). The novelty of this study lies in the combination of methodological and applied elements that have not previously been explored in buffalo-milk matrices. In particular, we adapted a droplet digital PCR assay to buffalo milk, a complex and fat-rich substrate for which no validated molecular protocol for the detection of *Cryptosporidium* spp. and *Giardia duodenalis* has been available. Building on this adaptation, we performed a comprehensive validation in accordance with ISO 16140-2, assessing specificity, sensitivity, repeatability, reproducibility, and recovery parameters that have never been systematically evaluated for protozoan detection in this matrix. Finally, we applied the validated assay to field buffalo-milk samples, generating the first ddPCR-based prevalence data for these protozoa in dairy buffalo farms and providing new insight into contamination dynamics within a high-value dairy sector. In line with international validation frameworks (EURACHEM, 2025; ISO 16140-1:2016; ISO 16140-2:2016), the method demonstrated excellent analytical performance, with high sensitivity, specificity, and robust repeatability and reproducibility. The extraction protocol yielded recovery efficiencies of 61% for *Giardia duodenalis* and 55% for *Cryptosporidium* spp. These values are consistent with those reported for direct DNA spiking experiments in complex food matrices, where losses are typically attributable to matrix-associated inhibitors, DNA adsorption, and partial extraction inefficiencies rather than lysis constraints (Barrera et al., 2024). These results indicate that the extraction and dd-PCR workflow provides reliable quantification of protozoan DNA in buffalo milk, although matrix effects may still influence absolute recovery. The dd-PCR assay reliably detected target DNA down to 1 gc/μL, with all replicates positive across the 1–100 gc/μL range, indicating that the true LOD₉₅ lies below the lowest concentration tested. This high analytical sensitivity reflects the intrinsic advantages of ddPCR technology, including reaction partitioning, absolute quantification, and improved tolerance to inhibitory compounds. In accordance with ISO 16140 (ISO 16140-2:2016), the specificity value reported here reflects the performance of the assay against the panel of negative buffalo milk samples and non-target DNA included in the validation study. The selection of non-target organisms for the specificity panel was based on their relevance to dairy production environments. *Escherichia coli*, *Staphylococcus aureus*, and *Listeria monocytogenes* are common bacterial contaminants associated with fecal matter, environmental hygiene, and raw-milk handling. Likewise, *Toxoplasma gondii*, *Neospora caninum*, and *Eimeria* spp. are protozoa frequently detected in livestock systems and

Table 4
Coefficient of variation (CV%) in samples across parasite concentration levels (100, 10, and 1 gc/μL).

<i>Giardia duodenalis</i>				<i>Cryptosporidium</i> spp			
100 gc/μl	10 gc/μl	1 gc/μl	Negative	100 gc/μl	10 gc/μl	1 gc/μl	Negative
61,2	5,4	0,78	not detected	55,2	5,1	0,62	not detected
59,8	5,1	0,65	not detected	54,6	4,8	0,58	not detected
60,5	5,6	0,92	not detected	56,1	5,3	0,71	not detected
62	5,3	0,71	not detected	53,9	4,9	0,55	not detected
60,9	5,5	0,88	not detected	55,7	5,2	0,69	not detected
59,5	5	0,63	not detected	54,1	4,7	0,51	not detected
61,7	5,7	0,95	not detected	56,4	5,4	0,75	not detected
60,2	5,2	0,69	not detected	53,8	4,9	0,48	not detected
62,4	5,6	1,02	not detected	55,9	5,2	0,66	not detected
60	5,3	0,74	not detected	54,4	5	0,59	not detected
Mean							
60,82 ± 0,99	5,37 ± 0,23	0,80 ± 0,14		55,01 ± 0,97	5,05 ± 0,23	0,61 ± 0,09	
CV							
1,63%	4,28%	17,5%		1,76%	4,55%	14,75%	
IC95%	[0,69, 1,00]				[0,69, 1,00]		

Table 5Intraday precision for *Giardia duodenalis* and *Cryptosporidium* spp. (three runs, five replicates each).

	<i>Giardia duodenalis</i>			<i>Cryptosporidium</i> spp		
	100 gc/μl	10 gc/μl	1 gc/μl	100 gc/μl	10 gc/μl	1 gc/μl
CV%	60.87 ± 0.88	5.36 ± 0.22	0.80 ± 0.14	55.1 ± 0.97	5.05 ± 0.23	0.61 ± 0.12
	1.4%	4.1%	17.0%	1.8%	4.5%	19.0%

Table 6Interday precision for *Giardia duodenalis* and *Cryptosporidium* spp. (three days, same operator).

	<i>Giardia duodenalis</i>			<i>Cryptosporidium</i> spp		
	100 gc/μl	10 gc/μl	1 gc/μl	100 gc/μl	10 gc/μl	1 gc/μl
CV%	60.7 ± 1.09	5.32 ± 0.27	0.79 ± 0.16	55.14 ± 1.09	5.04 ± 0.27	0.61 ± 0.14
	1.8%	5.1%	20.0%	2.0%	5.4%	26.0%

Table 7

Parasite-positive samples.

Sample	DATA	<i>Giardia duodenalis</i>		<i>Cryptosporidium</i> spp.	
		dd-PCR	gc/μl	dd-PCR	gc/μl
1	02/08/23	-	-	+	0,9
2	02/08/23	-	-	-	-
3	02/08/23	+	1,3	-	-
4	02/08/23	-	-	-	-
5	02/08/23	-	-	-	-
6	02/08/23	-	-	-	-
7	02/08/23	-	-	-	-
8	02/08/23	-	-	-	-
9	02/08/23	-	-	-	-
10	02/08/23	-	-	-	-
11	02/08/23	-	-	-	-
12	02/08/23	-	-	-	-
13	02/08/23	-	-	-	-
14	14/12/23	-	-	-	-
15	14/12/23	-	-	-	-
16	14/12/23	-	-	-	-
17	14/12/23	-	-	-	-
18	14/12/23	-	-	-	-
19	14/12/23	+	0,19	-	-
20	14/12/23	-	-	-	-
21	14/12/23	-	-	-	-
22	14/12/23	-	-	-	-
23	14/12/23	-	-	-	-
24	14/12/23	-	-	-	-
25	14/12/23	-	-	-	-
26	14/12/23	-	-	-	-
27	14/12/23	-	-	-	-
28	14/12/23	+	0,33	-	-
29	27/05/24	-	-	+	1,7
30	27/05/24	+	1,6	+	1,2
31	27/05/24	+	2,4	-	-
32	27/05/24	+	2,6	+	6,9
33	27/05/24	+	2,9	-	-
34	27/05/24	+	1,9	-	-
35	27/05/24	+	3	+	2,7
36	27/05/24	+	3,6	-	-
37	27/05/24	-	-	-	-
38	27/05/24	+	3,7	-	-
39	27/05/24	-	-	-	-
40	27/05/24	-	-	-	-
41	27/05/24	+	1,3	+	0,7
42	27/05/24	+	0,45	-	-
43	27/05/24	-	-	+	0,9
44	27/05/24	+	0,65	+	1,1
Prevalence (%)		31.8%		18.2%	
IC 95%		18.1% – 48.1%		8.2% – 32.7%	

may coexist in the same production settings. Including these organisms ensured that the specificity assessment reflected realistic potential interferents encountered in buffalo-milk matrices. ISO 16140 (ISO 16140-2:2016) states that specificity estimates are conditional on the

breadth and representativeness of the tested panel; therefore, the observed 100% specificity should be interpreted within the limits of the organisms and matrices evaluated and does not exclude the possibility of rare cross-reactivity with untested species. The absence of false-positive droplets further supports the assay's specificity. Precision analysis confirmed the robustness of the method, with low CV values at moderate and high concentrations and acceptable variability at 1 gc/μL, which is expected for assays operating near the limit of detection. The operator-independent nature of ddPCR enhances its applicability for routine diagnostic use in food-safety laboratories. This study assessed analytical sensitivity and specificity but did not evaluate diagnostic sensitivity or specificity, as field samples were not analyzed using a validated reference method. Therefore, the diagnostic performance of the ddPCR assay on raw buffalo milk remains undetermined. Interday reproducibility was evaluated using DNA aliquots prepared immediately after extraction and stored at −20 °C in AE buffer to prevent degradation associated with repeated freeze–thaw cycles. Although a specific degradation test was not performed, the consistent amplification profiles and comparable gene-copy numbers obtained across the three independent days suggest that the DNA remained stable under the storage conditions adopted. Application of the assay to field samples revealed the presence of *Giardia duodenalis* in 31.8% of buffalo milk samples and *Cryptosporidium* spp. in 18.2%. These prevalence values are higher than those typically reported with microscopy or conventional PCR, likely reflecting the superior analytical sensitivity of ddPCR, which enables the detection of low-abundance targets. However, ddPCR detects DNA irrespective of organism viability; therefore, the presence of protozoan DNA does not necessarily indicate the presence of infectious (oo)cysts. Future studies incorporating viability-based approaches, such as cell culture assays, or RNA-based detection methods are needed better to assess the actual risk of transmission through raw milk. Although an increase in detections was observed in May, ISO 16140 (ISO 16140-2:2016), emphasizes that an adequate number of test portions and representative sampling must support conclusions. In this study, only 44 samples were collected at three discrete time points from two farms, which does not fulfill ISO 16140 requirements for establishing temporal trends. Therefore, the observed increase should be interpreted as a preliminary indication rather than evidence of a confirmed seasonal contamination pattern. This trend is consistent with epidemiological data from temperate regions, where the prevalence of *Cryptosporidium* spp. and *Giardia duodenalis* typically rises in late spring and peaks during summer and early autumn. Several factors may contribute to this seasonal increase: environmental variables such as temperature, humidity, rainfall, and solar radiation, which influence (oo)cyst survival and dissemination (Wang et al., 2023); management-related factors, including calving and weaning periods, which are associated with increased oocyst shedding (Paraud & Chartier, 2012); and agricultural practices typical of late spring, such as manure spreading and the use of surface water for irrigation, which may enhance environmental

contamination (Tram et al., 2022). These findings underscore the importance of implementing targeted surveillance and preventive measures during high-risk periods.

5. Conclusion

This study developed and validated a dd-PCR assay for the detection of *Cryptosporidium* spp. and *Giardia duodenalis* in buffalo milk. The method demonstrated excellent analytical performance and represents a significant advancement for protozoan detection in dairy matrices. Application of the assay to buffalo milk samples from two provinces of the Campania Region revealed the presence of both protozoa, with a pronounced seasonal peak in May (this trend should be interpreted with caution due to the limited dataset). These findings underscore the influence of environmental and management factors on contamination dynamics and highlight the need for enhanced surveillance during late spring. This study has several methodological limitations. The sample size was limited, and parasite viability was not assessed, which restricts interpretation of the public-health relevance of the detected contamination. Moreover, the molecular assay employed enables detection of *Cryptosporidium* spp. and *Giardia duodenalis*, but it does not discriminate among species, genotypes, or assemblages; therefore, zoonotic potential cannot be inferred. Finally, environmental drivers underlying seasonal contamination patterns were not investigated. Future research should expand sample size, incorporate viability assays, apply sequencing-based characterization, and examine environmental determinants to support more targeted risk-mitigation strategies.

CRediT authorship contribution statement

Andrea Mancusi: Formal analysis, Investigation, Data curation; **Yolande Thérèse Rose Proroga:** Methodology, Resources, Supervision, Project administration, Funding acquisition. **Irene Dini:** Writing—original draft preparation, Data curation, Writing—review and editing, Visualization. **Carlo Grassi:** investigation; **Esterina De Carlo:** investigation; **Orlando Paciello:** review and editing; **Gabriele Di Vuolo:** investigation; **Antonio Bosco:** investigation; **Laura Rinaldi:** investigation; **Giovanna Cappelli:** Formal analysis, Investigation, Data curation

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Giovanna Cappelli reports financial support was provided by Italian Ministry. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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