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Current research trends towards the control of protozoans in foods

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Abstract
Protozoan parasites such as *Cryptosporidium* spp., *Giardia duodenalis*, *Toxoplasma gondii* and *Cyclospora cayetanensis* remain difficult-to-control hazards in food due to environmental persistence, low infectious doses, and the interpretability gap between nucleic acid detection and infectivity. This review synthesizes 4-year research trends shaping protozoan control in food systems, focusing on three critical pillars: matrix-adapted front-end processing (concentration, lysis, inhibitor management); inhibitor-resilient quantification; and sequencing-based attribution for outbreak investigation and source tracking. Recent benchmarking across wastewater, the water–soil–produce nexus, and food-relevant matrices repeatedly indicates – depending on matrix and study design – that upstream workflow steps often dominate analytical sensitivity and reproducibility. Accordingly, tiered analytical strategies are emerging in which the quantitative polymerase chain reaction (PCR) technique supports scalable screening, droplet digital PCR is used for decision-grade confirmation/quantification under inhibition and low-template conditions, and targeted sequencing or metagenomics is deployed selectively for traceback and contextual investigation. We integrate these developments into an actionable control framework that links prevention at the water–soil–plant interface with tiered analytics and viability-aware interpretation of post-intervention results. Research priorities ahead include harmonized performance reporting (recovery, inhibition controls, limit of detection/quantification), transparent endpoint hierarchy for intervention claims (detectability versus viability/infectivity), and interoperable sequence databases to enable cross-laboratory attribution and program-level learning. The field is moving from "can we detect?" towards "can we decide? – requiring reproducible front-end processing, inhibitor-resilient quantification, interoperable attribution resources, and endpoint discipline for intervention efficacy claims.

Introduction
Foodborne protozoan parasites are persistent hazards with disproportionate public-health impact relative to their typically low concentrations in foods. Controlling them is complicated due to the robustness of their environmental stages (cysts/oocysts), their low infectious doses, heterogeneous contamination in large batches, and analytical constraints in matrices rich in inhibitors. In this context, "control" should be defined operationally as an integrated pipeline spanning prevention, detection/quantification, attribution, and intervention validation.

Prevention focuses on minimizing contamination through effective pre-harvest and post-harvest barriers that interrupt transmission pathways from the environment or hosts to food. Detection and quantification rely on matrix-adapted analytical workflows whose performance characteristics. When an in-depth investigation is required, source attribution or traceability can be achieved through sequencing-based typing, providing the discriminatory resolution necessary to identify contamination origins and routes. Finally, intervention validation should employ evaluation criteria that consider parasite infectivity or viability, ensuring that post-treatment positive results are not misinterpreted and that control measures are accurately assessed in terms of public-health relevance.

The objective of this work is to conduct a narrative review that synthesizes recent methodological and applied evidence across foods and connected reservoirs (water, soil, wastewater). We position wastewater-based epidemiology (WBE) and environmental monitoring primarily as risk intelligence for food-control programs – supporting targeted sampling and hypothesis generation – rather than as direct evidence of contamination in specific food lots (FAO, 2025; Puchades-Colera *et al*., 2025).

In this review, "decision-grade" evidence refers to results that are auditable, comparable, and actionable in food-control contexts (*e.g*., routine monitoring, verification, incident response, and intervention assessment). In low-template and inhibitor-rich matrices, interpretability depends at least as much on upstream workflow performance and decision logic as on detection chemistry alone.

Accordingly, we address five review questions: i) Which upstream workflow steps most consistently constrain sensitivity and reproducibility across foods and connected reservoirs? ii) Under which conditions does droplet digital polymerase chain reaction (ddPCR) meaningfully improve interpretability relative to quantitative PCR (qPCR)? iii) When is sequencing most efficient and justifiable as a Tier 2 attribution tool? iv) What minimum controls or metrics are required to interpret positive and negative results as decision-grade? v) Which endpoints support defensible intervention claims (detectability vs viability vs infectivity)?

Methods (evidence identification and selection)

We conducted a structured narrative review focusing on research developments from a January 2022 to 24 February 2026 in protozoan control in foods and food-relevant environments. Although this is a structured narrative review (not a systematic review), we adopted proportionate transparency to reduce selection bias and improve auditability, consistent with narrative review quality guidance and literature-search reporting expectations where applicable (Baethge *et al*., 2019; Rethlefsen *et al*., 2021). Database-specific search strategies (including at least one full reproducible strategy), last-search dates, and information sources beyond databases are documented in *Supplementary Material S1*. A register of included studies with core metadata (*i.e*., matrix, organism, design class, and decision-grade reporting elements) is provided in *Supplementary Material S2*.

Sources

Primary studies and authoritative guidance documents were prioritized. Preprints were included only when they provided clearly described methods with plausible near-term relevance to surveillance practice, and were interpreted as non-peer-reviewed evidence (Torii *et al*., 2025).

Search strategy (structured, non-exhaustive)

Literature searches were performed in Scopus, Web of Science, and PubMed using combinations of terms including protozoa (*Cryptosporidium*, *Giardia*, *Toxoplasma*, *Cyclospora*), food and other sources (produce, lettuce, berries, shellfish, oysters, wastewater, irrigation), analytical methods (ddPCR, qPCR, metagenomics, nanopore, targeted amplicon sequencing, genotyping, and viability [propidium monoazide quantitative qPCR (PMA-qPCR), ethidium monoazide qPCR (EMA-qPCR), real-time qPCR (RT-qPCR)]. Reference lists of high-relevance papers and agency documents were screened to capture additional primary sources and method evaluations. Database searches were last updated on 24 February 2026; deduplication and screening logic are summarized in *Supplementary Material S1*.

Eligibility criteria

The criteria for inclusion were defined as studies addressing food matrices, shellfish, irrigation/source waters, wastewater, or the farm nexus (water-soil-produce) with explicit relevance to contamination prevention, detection, attribution, or intervention validation; comparison of methods or optimized workflows reporting at least one decision-grade performance element [*e.g*., recovery, inhibition controls/mitigation, limit of detection (LOD), limit of quantification (LOQ)], replication structure, turnaround time, field applicability); or agency documents relevant to outbreak investigation, surveillance frameworks, or risk assessment and control programs.

Exclusion

Two types of studies were excluded: studies lacking sufficient methodological detail to assess reproducibility or analytical validity, and studies not plausibly transferable to food-control decisions (*e.g*., purely clinical diagnostics without linkage to exposure assessment, attribution, or surveillance design).

Full search strategies and an included-study register are provided in *Supplementary Materials S1 and S2*.

A control-point framework for protozoa in foods

Figure 1 summarizes protozoan control as a connected, One Health pipeline. The framework is intended to support decision-making in three recurring situations: i) routine monitoring, which involves selecting fit-for-purpose workflows given matrix inhibition and expected low loads; ii) incident response, which entails rapid confirmation and quantification followed by attribution when warranted; and iii) intervention assessment, which focuses on distinguishing reductions in detectability from reductions in infectivity. Operationally, it motivates layered testing strategies – screening by qPCR, escalation to ddPCR where inhibition undermines interpretability, and targeted amplicon sequencing (TAS) or metagenomics where traceback or multi-hazard context is required – while keeping prevention and viability-aware interpretation as first-class design elements (Hachimi *et al*., 2024; Ahlinder *et al*., 2022; Naushad *et al*., 2025; Schipper *et al*., 2025).

Analytical control: detection, quantification and attribution

This section synthesizes recent advances in decision-grade analytics for the control of foodborne protozoa and is structured around three complementary operational objectives. It first addresses screening, which emphasizes rapid and highly sensitive detection of target parasites to enable early decision-making. It then focuses on quantification and confirmation, ensuring that analytical results yield credible positives and interpretable negatives even in the presence of matrix-related inhibition. Finally, it examines attribution and traceback, where methods offering sufficient discriminatory power are applied to support epidemiological and food-chain investigations.

Reasons why front-end sample preparation dominates performance

Across wastewater, farm-nexus matrices, and shellfish, method comparisons repeatedly indicate – depending on matrix and study design – that analytical sensitivity and reproducibility are frequently determined by upstream workflow steps – concentration, lysis and inhibitor management – rather than amplification chemistry alone (Fitri *et al*., 2022; Hachimi *et al*., 2024; Puchades-Colera *et al*., 2025; Schipper *et al*., 2025; Rodriguez *et al*., 2026). For protozoa, this is expected: (oo)cysts can be hard to disrupt, target DNA is sparse, and co-extracted matrix components often suppress amplification.

Recent benchmarking efforts further highlight the matrix-dependence of analytical performance and the existence of critical "bottleneck" steps. In wastewater benchmarking, centrifugation delivered the highest oocyst recoveries; bead beating improved DNA recovery relative to freeze–thaw; and a broadly conserved 18S qPCR target showed improved analytical sensitivity over the *Cryptosporidium* outer wall protein (COWP) gene detection in that context (Hachimi *et al*., 2024).

Studies on hospital and urban wastewater surveillance indicate that aluminium-based adsorption–precipitation combined with freeze–thaw cycles and magnetic bead extraction improved recoveries and supported a feasible WBE protocol (Puchades-Colera *et al*., 2025).

Meanwhile, comparative evaluations in oysters and seawater revealed that extraction efficiency differs across matrices: bead beating was advantageous for seawater, whereas freeze–thaw was comparable or better for some targets in oysters, reinforcing the need for matrix-specific optimization rather than one-size-fits-all protocols (Rodriguez *et al*., 2026).

From a control perspective, routine monitoring programs benefit most from investing in standardized front-end processing steps, including robust recovery and inhibition controls. Such optimization generally produces greater improvements in data interpretability and inter-laboratory transferability than incremental assay changes.

Quantitative PCR vs. droplet digital PCR under inhibition and low-template conditions
Real-time PCR remains the dominant Tier 1 tool for screening, but its interpretability can degrade when inhibitors are co-extracted or when template abundance approaches the detection limit (Lotz *et al*., 2025). The ddPCR technique is increasingly positioned as a practical Tier 1b option for inhibitor-rich matrices because partitioning can mitigate inhibition effects and enables absolute quantification without standard curves.

In a study spanning water, soil, and fresh produce, ddPCR was less prone to PCR inhibition than real-time PCR; and – critically for control decisions – identified field positives missed by qPCR: none of 210 environmental samples tested positive by real-time PCR, while ddPCR detected *Cryptosporidium* in 13.6% of water, 23.3% of soil, and 34.7% of produce samples (Schipper *et al*., 2025). These differences can materially affect the interpretability of negative results, conditional on effective sample volume (ESV), droplet counts, replication rules, and explicit inhibition/recovery controls – elements that should be transparently reported for decision-grade use (Box 1) (dMIQE Group and Huggett, 2020).

From a control perspective, a pragmatic and tiered workflow involves using qPCR for high-throughput screening with explicit inhibition controls, followed by escalation to ddPCR for confirmatory quantification when inhibition risk is high, when results are borderline or indicate low loads, or when decision-grade evidence is needed for applications such as verification, trend analysis, or intervention evaluation (Schipper *et al*., 2025).

Metagenomic next generation sequencing as a hypothesis-minimizing platform
Metagenomic next generation sequencing (mNGS) is increasingly assessed as an enabling platform for multi-hazard detection and contextual inference in complex foods. For protozoa, the central constraint remains the "needle-in-a-haystack" problem: target DNA is sparse and embedded within abundant host/food DNA backgrounds, while (oo)cysts can be difficult to lyse.

A representative workflow for leafy greens combined rapid lysis (3 min), acetate precipitation, whole-genome amplification, and nanopore sequencing, enabling consistent identification of as few as 100 *C. parvum* oocysts in 25 g of lettuce and simultaneous detection of multiple protozoa (including *C. hominis*, *C. muris*, *G. duodenalis*, and *T. gondii*) under operationally realistic laboratory conditions (Naushad *et al*., 2025). From a control standpoint, the key lever is not sequencing alone, but front-end sample processing (lysis, enrichment, inhibitor management) aligned with the broader message from wastewater and nexus benchmarking (Hachimi *et al*., 2024; Puchades-Colera *et al*., 2025).

The strengths of mNGS lie in its capacity for broad detection without preselecting targets, supporting multi-agent screening and exploratory investigation; and in its potential to integrate protozoan findings with microbial context — for instance, by supporting source hypotheses when integrated into source-tracking frameworks (Ahlinder *et al*., 2022). However, the technique also faces notable constraints, including the need for infrastructure and bioinformatic standardization such as rigorous contamination controls and curated reference databases. Moreover, interpretation remains limited because nucleic-acid detection alone does not confirm infectivity, making viability-aware interpretation a critical requirement (see section "Viability-aware inference and common pitfalls").

Targeted amplicon sequencing for traceback and source attribution
TAS complements mNGS by prioritizing discriminatory loci to enable higher-resolution typing at lower cost. In practice, TAS is best positioned as a Tier 2 method – used after sensitive screening/confirmation – when attribution is required for outbreak investigation and root-cause analysis.

For *Cyclospora cayetanensis*, genotyping panels and expanded locus sets are increasingly integrated into outbreak and traceback frameworks (FDA, 2023; National Advisory Committee on

Microbiological Criteria for Foods, 2023; Leonard *et al*., 2023; Leonard *et al*., 2024). The control value is greatest when contamination levels are low and when investigators need shared, interoperable genetic fingerprints to compare lots, farms, or regions.

From a control positioning standpoint, TAS can be deployed after qPCR/ddPCR confirmation to maximize efficiency and reduce "wasted reads" on background DNA. To ensure cross-laboratory comparability and consistent interpretation, standardized loci and curated reference datasets should be prioritized.

Complementary approaches: automation and biosensing

Where sequencing and advanced PCR are impractical at scale, complementary approaches may support triage, screening, or early warning – particularly upstream of foods, such as in source waters. Examples include AI-assisted microscopy (*e.g*., YOLO-based object detection), which illustrates a pathway for scalable image-based screening in environmental monitoring contexts (Kahraman *et al*., 2024); and Label-free microfluidic impedance flow cytometry, which shows potential for continuous monitoring and discrimination of *Giardia* cysts and *Cryptosporidium* oocysts in flowing water, including natural-water suspensions (Peng *et al*., 2025).

Control implication: these technologies are best framed as upstream intelligence tools that complement, rather than replace, decision-grade confirmation workflows for food-lot decisions.

Practical reporting standard for control programs

A schema of minimum information for decision-grade protozoa detection/quantification (foods and food-relevant matrices) is proposed, and is intended to be interoperable with established minimum-information guidance for PCR-based experiments, and should be mapped to MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) and dMIQE (Minimum Information for Publication of Quantitative Digital PCR Experiments) domains in *Supplementary Material S3* to support auditability and comparability (Bustin *et al*., 2009; dMIQE Group and Huggett, 2020).

Sampling and design involve defining the matrix and analytical unit, including the lot, subsample, and compositing strategy, as well as the mass or volume processed. The sampling context—whether pre-harvest or post-harvest and whether involving water, soil, or food—is specified along with the number of independent samples analyzed.

Process performance encompasses recovery control, specifying the process spike (organism or standard used), the method of recovery calculation, and the recovery distribution expressed as mean, median, and range or confidence interval. Inhibition control is evaluated through the use of an internal amplification control or equivalent, including explicit inhibition mitigation steps and decision rules applied for re-extraction or retesting.

Analytical definition includes the assay target or targets and their rationale (for example, 18S versus species-specific targets), including primer and probe sequences and reaction conditions. Positivity criteria are described in terms of Ct or threshold rules for qPCR and droplet thresholds for ddPCR, together with the replicate logic applied. Definitions of LOD and LOQ are provided along with the replication structure, with technical and biological replicates reported separately

Interpretation and (if applicable) viability include an interpretation statement distinguishing detection from infectivity and, where viability can be inferred, the exact method and controls supporting that inference (see section "Viability-aware inference and common pitfalls").

Reproducibility and transparency encompass protocol availability, specifying whether a versioned standard operating procedure (SOP) or sufficient methodological detail is provided to enable replication, and, for sequencing studies, the pipeline parameters and reference databases used. Data and metadata deposition are indicated where feasible, including negative controls and batch structure.

Intervention technologies and validation endpoints

Analytical advances have improved detection and attribution, but control ultimately requires interventions that reduce exposure and/or infectivity. For protozoa, intervention claims are frequently limited by endpoint ambiguity (DNA detectability vs infectivity) and by matrix realism (organic load, surface complexity, and lot heterogeneity). This section summarizes the intervention landscape and proposes validation practices aligned with decision-grade control.

Viability-aware inference and common pitfalls

Molecular detection supports inference about detectability but does not establish viability or infectivity. Therefore, any "inactivation" or "efficacy" claim must be tied to infectivity endpoints where feasible, or to validated viability proxies with explicit controls and uncertainty reporting. DNA-only outcomes can support "reduction in detectability" but are insufficient as a sole basis for "inactivation".

Intervention landscape: where the evidence is converging

The most consistent trend is a shift towards physical and non-thermal interventions that preserve product quality while targeting (oo)cyst integrity. Current evidence is strongest when interventions are evaluated under food-relevant conditions and interpreted using viability-aware endpoints.

Cold atmospheric plasma (CAP) has been evaluated for protozoan inactivation/viability impacts on produce/herb surfaces, aligning with "waterless" intervention concepts and the practical need for surface treatments (Craighead *et al*., 2020).

High-pressure processing (HPP) has a longstanding evidence base for inactivating protozoa in food-relevant matrices (including fruits), with a strong rationale for quality-preserving control where heat is undesirable (Lindsay *et al*., 2008).

Pulsed electric fields (PEF) are being explored primarily for liquid matrices (water/juices), with potential applicability to process water and beverage contexts when validated with appropriate endpoints (Berzosa *et al*., 2025).

Control implication: for protozoa, intervention selection should be matrix- and process-specific: surface-focused approaches (*e.g*., CAP) are conceptually distinct from bulk/volumetric approaches (HPP/PEF), and the validation design should reflect the dominant mechanism and exposure pathway.

Endpoint discipline: what "works" must be measurable

Because nucleic acids can persist beyond loss of infectivity, intervention success cannot be inferred from DNA detection alone. Therefore, intervention validation should explicitly define (and justify) the primary endpoint.

Infectivity endpoints are preferred when feasible using cell culture or animal models where ethically and practically justified. Validated viability proxies are acceptable when infectivity assays are impractical, such as RT-qPCR approaches or other viability-linked markers, provided that explicit controls and uncertainty reporting are included (Liang and Keeley, 2012).

DNA-only outcomes are insufficient as a sole endpoint for efficacy but can support "reduction in detectability" rathen than "inactivation".

This endpoint hierarchy should be paired with inhibitor-aware quantification. In complex matrices, ddPCR can provide more credible post-intervention quantification than qPCR under inhibition, supporting decision-grade interpretation of low-load signals when ESV and replication rules are explicit (Schipper *et al*., 2025; dMIQE Group and Huggett, 2020).

Coupling interventions to tiered analytics (recommended design pattern)

A practical pattern for intervention studies and control verification consists of specific methods of quantification pre-intervention, post-intervention and escalation for attribution. The assessment pre-

intervention should quantify baseline burden using a workflow with explicit recovery and inhibition controls (Tier 1 screen, Tier 1b quantification if needed); whereas the assessment post-intervention should quantify residual signal with the same controls and explicitly state whether the outcome is "detectability reduction" or "viability/infectivity reduction". Escalation for attribution should be conducted only when an incident response requires traceback (Tier 2: TAS or mNGS), recognizing that attribution workflows are not primarily designed to validate inactivation. This coupling avoids over-claiming efficacy while enabling consistent comparisons across interventions and matrices.

Practical recommendations for control programs

In control programs and study designs targeting the measurement of the efficacy of interventions, five practical recommendations could be derived from the present review. First, use matrix-realistic conditions, considering organic load, surface complexity, contact time, temperature, and lot heterogeneity. Second, treat recovery and inhibition as first-class design elements. Third, predefine the decision claim —whether the focus is on detectability reduction, viability reduction or infectivity reduction. Fourth, prefer tiered analytics: qPCR for screening and ddPCR for confirmatory quantification when inhibition or low-load is expected and decision thresholds are stringent (Schipper *et al*., 2025). Finally, report uncertainty explicitly, especially when viability inference relies on proxies.

Comparative snapshot of key primary studies (control-relevant)

Table 1 summarizes the primary studies emphasized in this review, organized to support control decisions rather than purely methodological description. In particular, it highlights the operational tier each study best informs — whether screening or quantification, attribution, or upstream intelligence — the dominant bottleneck, such as concentration, lysis or inhibition. and the degree of transferability to foods, distinguishing between direct food matrix evidence and upstream reservoir intelligence. Studies in clinical settings are included only where they clarify implementation constraints and interpretation principles relevant to outbreak linkage and burden estimation, but they are not treated as direct evidence for food-lot control.

Synthesis: an actionable, tiered control workflow

Figure 1 provides a system-level view of protozoan control across the water–soil–plant–food continuum, emphasizing prevention, tiered analytics, and viability-aware interpretation. Building on that framework, Figure 2 operationalizes method selection as a decision workflow, while Table 2 summarizes a compact mapping between objectives, matrices, recommended tiers/tools, and minimum critical controls.

A tiered decision logic (screening to quantification to attribution)

A pragmatic tiered strategy separates rapid screening from decision-grade quantification and from attribution tasks.

Tier 1**,** screening, includes qPCR with explicit inhibition controls, and prioritization of matrix-adapted concentration and extraction, where recovery and inhibition are first-class design elements.

Tier 1b, robust quantification encompasses ddPCR when inhibition risk is high or when low-load detection credibility is critical; for instance, in scenarios of verification, trend analysis or pre/post intervention comparisons (Schipper *et al*., 2025).

Tier 2, attribution and traceback should be carried out by TAS/genotyping for traceback when discriminatory resolution is needed – especially for *Cyclospora* (FDA, 2023; National Advisory Committee on Microbiological Criteria for Foods 2023; Leonard *et al*., 2023; Leonard *et al*., 2024); and by mNGS for broad detection and contextual source hypotheses in multi-hazard or exploratory investigations (Ahlinder *et al*., 2022; Naushad *et al*., 2025). Finally, Intervention validation involves

the quantification of pre-/post-intervention should be coupled with an endpoint hierarchy — infectivity where feasible or otherwise validated viability proxies. Furthermore, interpreting DNA-only signals as efficacy should be avoided.

Where wastewater-based epidemiology fits in food control

WBE is best positioned as risk intelligence to allocate monitoring effort and to evaluate watershed/sanitation dynamics, rather than as direct farm- or lot-level attribution (Hachimi *et al*., 2024; Puchades-Colera *et al*., 2025). In control programs, WBE signals can justify intensified sampling of upstream watersheds or targeted food testing during periods of elevated community burden, consistent with the prevention-centric framing in Figure 1.

Decision matrix for implementation

Table 2 condenses implementation choices into a decision matrix suitable for program design and study planning. The table highlights that, across matrices, the minimum decision-grade standard is defined less by amplification chemistry than by upstream process performance (recovery) and interpretability controls (inhibition assessment, replicate logic, and explicit positivity criteria).

Gaps and research priorities

Despite rapid progress in detection, quantification, and attribution, protozoan control in foods remains constrained by three recurring limitations: incomplete standardization of workflow performance reporting; endpoint ambiguity — specifically the distinction between detectability and infectivity; and limited interoperability of typing data and reference resources. Below, we aimed to translate these limitations into research priorities and measurable deliverables in the near future.

Standardized performance reporting and reference materials

Cross-study and cross-laboratory comparisons remain difficult because recovery, inhibition assessment, replicate structure, and LOD/LOQ are inconsistently defined and reported. The priority deliverables to enhance standardization include: i) the development of a minimum information standard for protozoa workflows — aligned with Section 5 and adopted across food, water, soil and produce studies; ii) the establishment of shared reference materials and process controls suitable for inhibitor-rich matrices (produce, shellfish, soils), enabling benchmarking beyond single-lab conditions; and iii) the adoption of consensus definitions for LOD/LOQ and positivity criteria that explicitly incorporate replication structure distinguishing between biological and technical replicates.

Viability and infectivity: harmonized endpoint hierarchy

Many studies still conflate nucleic-acid detection with inactivation, leading to over-interpretation of post-intervention positives/negatives. To counteract this problem, priority research should focus on establishing a harmonized endpoint hierarchy (infectivity > validated viability proxy > detectability) implemented as a reporting requirement for intervention studies. It should also emphasize standardized viability controls (positive/negative viability controls, time/temperature handling rules) and transparent uncertainty reporting when infectivity assays are impractical. In addition, comparative studies are needed to map how long DNA and RNA targets persist after loss of infectivity across representative food matrices and process conditions.

Tiered surveillance design: when to escalate from quantitative PCR to digital droplet PCR

Programs often deploy qPCR uniformly, even where inhibition and low-template conditions undermine interpretability. In order to determine under which circumstances to switch from qPCR to ddPCR, priority deliverables are the derivation of decision rules for escalation based on inhibition metrics,

matrix class, and decision context (routine monitoring vs verification vs incident response), as well as comparative cost–performance evaluations of qPCR/ddPCR strategies under realistic sampling designs (lot heterogeneity and composite sampling), reporting decision error rates (false negatives under inhibition).

Sequencing for traceback: interoperability, databases, and quality assurance

While TAS and mNGS are maturing, harmonization of loci/panels, reference datasets, and quality thresholds remains limited, constraining cross-jurisdiction attribution. To this respect, sequencing for traceback would benefit from standardized TAS panels (especially for *Cyclospora*) with clearly defined locus sets, QC metrics, and metadata requirements to support cross-laboratory comparability, interoperable reference databases and deposition workflows (sequence and minimal metadata), with transparent thresholds for calling true positives in low-load samples, and validation studies assessing reproducibility across laboratories and platforms, including bioinformatic pipeline sensitivity to parameters and database choice.

Metagenomic next generation sequencing operationalization: contamination control and decision thresholds

Routine mNGS adoption is limited by cost, complexity, and uncertain decision thresholds in sparse-target contexts. To improve the operationalization of mNGS, research should deliver: i) standard contamination-control schemas (including field blanks, extraction blanks and library blanks) and reporting norms for mNGS studies in foods and environmental reservoirs; ii) decision thresholds for detection calls that incorporate negative-control distributions and batch effects, enabling defensible interpretation in surveillance settings; and iii) workflow optimization studies quantifying how front-end processing (lysis, enrichment, or whole genome amplification [WGA]) shifts sensitivity trade-offs in food matrices.

Bridging upstream signals to food risk: from monitoring to quantitative microbial risk assessment-ready inference

WBE and environmental monitoring increasingly generate signals, but translation into food-risk decisions is not yet standardized. Priority research in this sense is to produce frameworks that map upstream signals (such as WBE, irrigation waters, and soil) to decision triggers for targeted food testing and preventive actions; quantitative microbial risk assessment integrations that explicitly incorporate inhibition-aware quantification uncertainty and lot heterogeneity; and longitudinal datasets linking upstream monitoring, weather/runoff events, and food contamination outcomes to calibrate prevention-oriented interventions.

Conclusions

Protozoan control in foods is increasingly shaped by methodological convergence and by a shift from "detection capability" to decision-grade control. Across food matrices and connected environmental reservoirs, recent benchmarking evidence consistently indicates that front-end processing—concentration, lysis efficiency, and inhibitor management—often dominates analytical sensitivity and reproducibility. This observation is not a technical footnote: it is a primary determinant of whether surveillance results are interpretable and transferable across laboratories and matrices.

A second convergence concerns quantification under inhibition. Where inhibitors and low-template conditions are common (soil, produce extracts, and some shellfish workflows), ddPCR is increasingly positioned as a practical escalation tool to improve interpretability under stringent decision thresholds. However, "negative" calls remain conditional on effective sample volume, replication structure, and transparent recovery/inhibition metrics.

Third, sequencing is maturing into a targeted instrument for attribution. mNGS can support multi-hazard detection and contextual investigation when coupled to matrix-adapted front-end processing, while targeted amplicon sequencing provides a pragmatic route to traceback-ready resolution—particularly for *Cyclospora*—when standardized loci and interoperable reference resources are available. These sequencing tools are most effective when deployed as Tier 2 methods following sensitive screening and credible quantification, rather than as stand-alone first-line tests.

Finally, progress in detection and attribution will not translate into control without endpoint discipline. Because nucleic acids can persist after loss of infectivity, intervention claims must distinguish reductions in detectability from reductions in viability or infectivity, and validation designs should explicitly prioritize infectivity endpoints where feasible or otherwise use validated viability proxies with transparent uncertainty. The control-point framework emphasizes this interpretability layer to avoid over- or under-reacting to post-intervention molecular signals.

Overall, the practical path forward is a coherent, prevention-centered and tiered approach: strengthen upstream barriers at the water–soil–plant interface; implement standardized, matrix-adapted workflows with explicit recovery and inhibition controls; escalate from qPCR to ddPCR when interpretability demands; and deploy sequencing strategically for attribution. Harmonized reporting, viability-aware inference, and interoperable sequence databases emerge as immediate priorities to enable true comparability and cross-jurisdiction learning.

As limitations of this review, authors point out that this synthesis is a structured narrative review and does not constitute an exhaustive census of all publications. Evidence remains heterogeneous across matrices and study designs, and decision-grade comparability is limited where recovery, inhibition, effective sample volume, LoD/LoQ, and replication rules are not reported consistently. *Supplementary Materials S1 and S2* are therefore essential for auditability and for appropriately weighting convergence claims.

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Online supplementary material

Supplementary Material S1. Search strategies, databases, last-search dates, deduplication and screening logic (PRISMA-S-style reporting).

Supplementary Material S2. Included-study register (matrix, organism, design class, tier, and decision-grade reporting elements).

Supplementary Material S3. Crosswalk to MIQE/dMIQE domains.

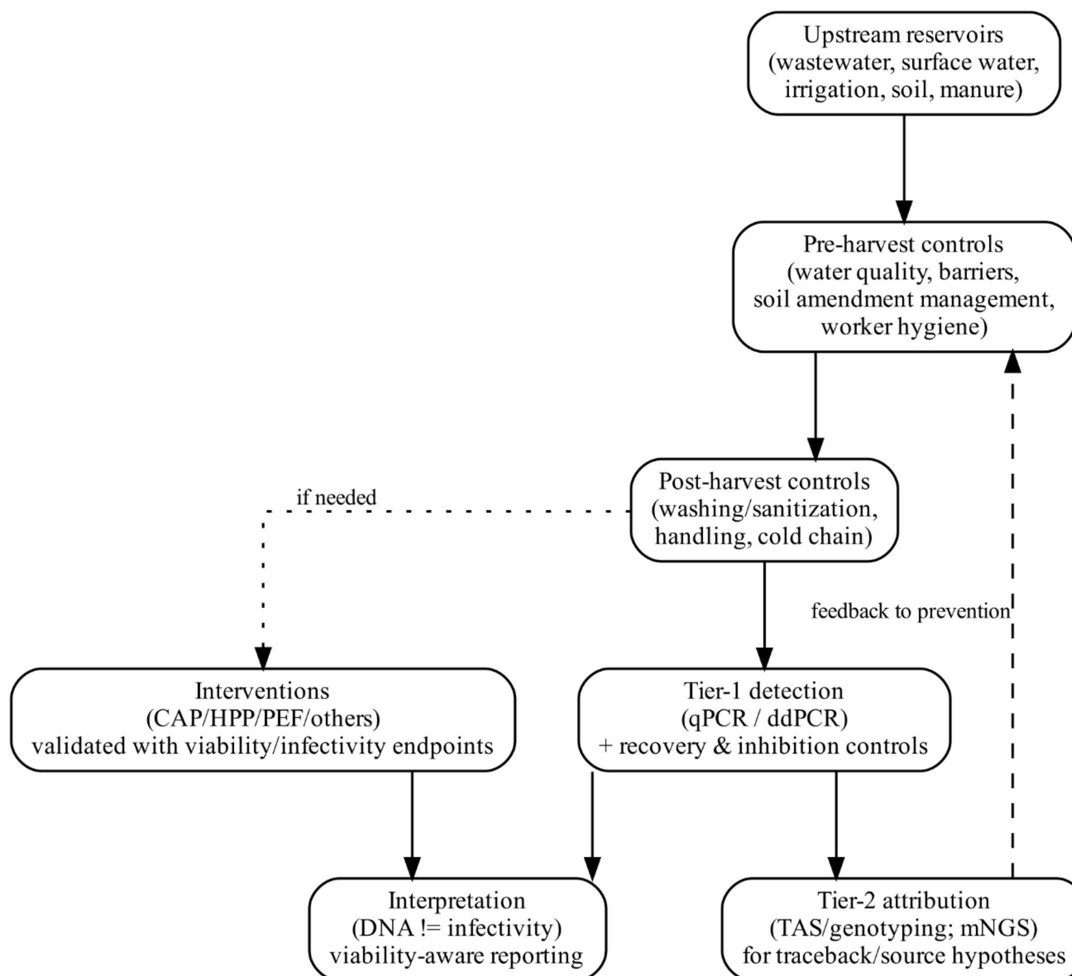

Figure 1. One Health control-point framework linking prevention, tiered detection, attribution, and intervention validation. CAP, cold atmospheric plasma; ddPCR, droplet digital polymerase chain reaction; HPP, high pressure processing; mNGS, metagenomic next generation sequencing; PEF, pulsed electric fields; qPCR, quantitative polymerase chain reaction; TAS, targeted amplicon sequencing.

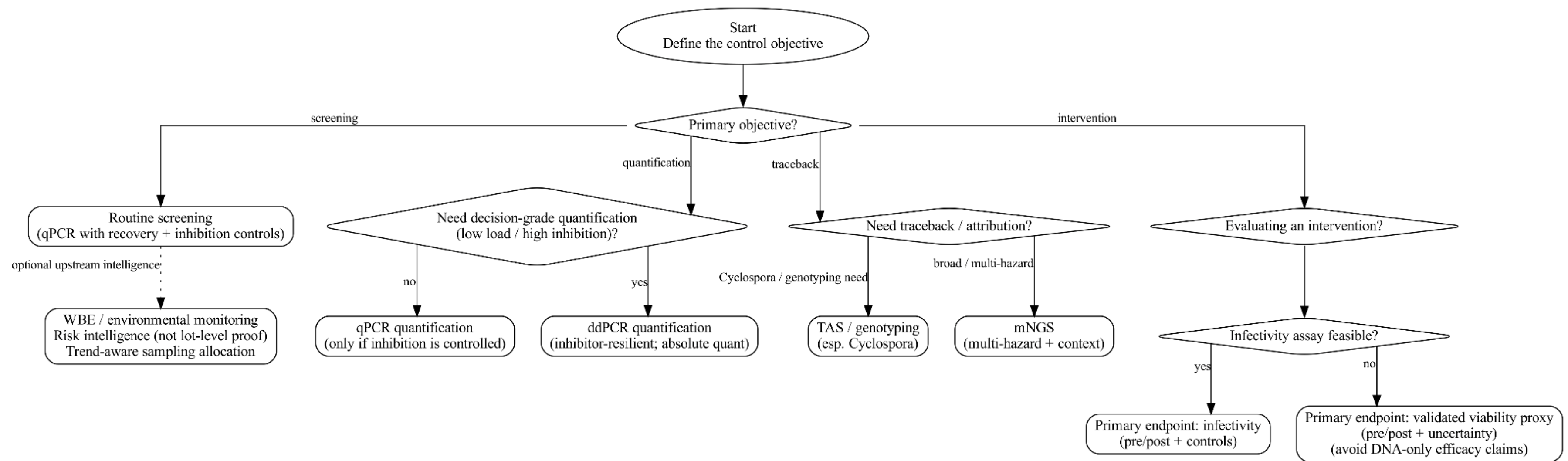

Figure 2. Tiered analytical decision workflow for protozoa control (screening, quantification, attribution, intervention validation). ddPCR, droplet digital polymerase chain reaction; mNGS, metagenomic next generation sequencing; qPCR, quantitative polymerase chain reaction; TAS, targeted amplicon sequencing; WBE, wastewater-based epidemiology.

Table 1. Evidence map of control-relevant primary studies and their operational implications*.

Study	Matrix / system	Primary control objective	Operational tier (typical use)	Main methodological lever / bottleneck	Decision-grade controls highlighted	Key control-relevant contribution
Ahlinder *et al*. (2022)	Outbreak-linked romaine lettuce; farm investigation	Prevention + traceback hypothesis strengthening	Tier 2 (contextual investigation)	Shotgun metagenomics + microbial source tracking	Integrating parasite detection with contextual microbial signatures	Shows how metagenomics/source tracking can strengthen upstream source hypotheses for prevention and incident response.
Rodriguez *et al.* (2026)	Oysters and seawater	Screening/quantification feasibility across matrices	Tier 1 / 1b (method selection by matrix)	Extraction method is matrix-dependent (freeze–thaw vs bead beating)	Matrix-specific optimization needs	Demonstrates that optimal extraction differs between seawater and oysters; supports matrix-adapted protocols for shellfish surveillance.
Hachimi *et al*. (2024)	Wastewater	Surveillance workflow optimization (WBE)	Tier 1 / 1b (upstream intelligence)	Concentration + lysis drive recovery; target affects sensitivity	Recovery benchmarking; assay comparison logic	Supports protocol choices where front-end steps dominate performance; provides comparative evidence for target selection.
Puchades-Colera *et al*. (2025)	Hospital + urban wastewater (WBE)	Risk intelligence + workflow benchmarking	Tier 1 / 1b (upstream intelligence)	Concentration + extraction benchmarking; field implementation	Workflow feasibility in field settings	Provides a feasible WBE workflow and prevalence signals useful for risk-aware monitoring (not lot-level proof).
Schipper *et al*. (2025)	Water–soil–produce nexus (farm context)	Decision-grade detection under inhibition	Tier 1b (confirmation/quantification)	ddPCR robustness under inhibition and low template	Inhibition-resilient quantification emphasis	Demonstrates ddPCR advantage in inhibitor-rich matrices; affects interpretability of negatives and sampling design.
Naushad *et al*. (2025)	Leafy greens (lettuce), spiking	Multi-protozoa detection in food matrix	Tier 2 (broad screening + context)	Front-end lysis/enrichment + WGA enable mNGS	Workflow components enabling sensitivity in food background	Shows feasible mNGS for multiple protozoa in leafy greens; front-end processing is the key lever.
Torii *et al.* (2025)	Environmental water (**preprint**)	"All-in-one" multi-pathogen monitoring concept	Tier 1 (upstream intelligence; exploratory)	Membrane adsorption + direct extraction; rRNA targeting	Preprint: interpret cautiously; needs independent validation	Illustrates integrated monitoring design; valuable concept but weight cautiously until peer reviewed.
Peng *et al.* (2025)	Flowing water (incl. natural water)	Early-warning / continuous monitoring	Upstream intelligence (triage/monitoring)	Microfluidic impedance flow cytometry	Complementary to molecular confirmation	Demonstrates label-free discrimination potential for continuous monitoring in source waters.
Kahraman *et al.* (2024)	Environmental monitoring	Screening automation	Upstream intelligence (triage)	AI-assisted microscopy (object detection)	Requires calibration/validation thresholds	Shows potential for scalable microscopy triage; not a substitute for confirmatory assays.
Lotz *et al.* (2025)	Field stool diagnostics (**clinical**)	Burden estimation; species differentiation	Contextual (linking exposure to burden)	qPCR implementation (operational feasibility)	Operational constraints; multiplexing considerations	Useful for interpretability and burden linkage; indirect for food matrices.
Fitri *et al.* (2022)	Review (**clinical**)	Diagnostic landscape (background rationale)	Contextual (rationale; not primary evidence)	Review of immunological + molecular diagnostics	Background rationale for molecular surveillance	Not direct food-matrix evidence; supports rationale for molecular methods.

(*) Tier 1 = screening; Tier 1b = inhibitor-resilient confirmation/quantification; Tier 2 = attribution/traceback and/or broad sequencing-enabled investigation; "Upstream intelligence" denotes evidence primarily supporting prevention-oriented monitoring (e.g., WBE/source waters) rather than direct food-lot decisions; Clinical studies are included for interpretability and operational constraints, not as direct matrix evidence for foods. AI, artificial intelligence; ddPCR, droplet digital polymerase chain reaction; mNGS, metagenomic next generation sequencing; qPCR, quantitative polymerase chain reaction; rRNA, ribosomal ribonucleic acid; WBE, wastewater-based epidemiology; WGA, whole genome amplification

Table 2. Decision matrix linking objectives, matrices, tiers, minimum controls, and interpretation limits.

Objective	Typical matrix	Recommended tier	Expected output	Minimum required controls	Interpretation limits
Routine screening	Fresh produce, irrigation water, shellfish extracts	Tier 1 (qPCR)	Detection / screening signal	Recovery control + IAC/IPC + LoD/LoQ definition + positivity rule	"Negative" is conditional on ESV, inhibition, and replication
Decision-grade confirmation/quantification	Inhibitor-rich matrices; borderline/low-load signals	Tier 1b (ddPCR or optimized qPCR)	Robust quantification + uncertainty	ESV + droplet/replicate rules + recovery + inhibition mitigation + blanks	ddPCR does not imply infectivity; still depends on extraction performance
Traceback/attribution	Outbreak investigations, source hypotheses	Tier 2 (TAS)	Genotype / multilocus profile	Marker QC (dropout thresholds) + controls + curated reference set	Comparability depends on shared panels/DB and metadata completeness
Contextual investigation/multi-hazard	Complex foods; exploratory One Health investigations	Tier 2 (mNGS)	Multi-agent detection + context	Extraction/library blanks + spike-ins + pipeline/DB versions + thresholds	Sparse targets; contamination/batch effects can dominate
Intervention validation	Pre/post treatment studies	Endpoint-tiered	Claim tier (detectability/viability/infectivity)	Endpoint hierarchy + appropriate controls + inhibitor-aware quantification	DNA-only different from inactivation; viability proxies require explicit uncertainty

DB, database; ddPCR, droplet digital polymerase chain reaction; ESV, exact sequence variance; IAC, internal amplification control; IPC, internal positive control; LoD, limit of detection; LoQ, limit of quantification; mNGS, metagenomic next generation sequencing; QC, quality control; qPCR, quantitative polymerase chain reaction; TAS, targeted amplicon sequencing; WBE, wastewater-based epidemiology.