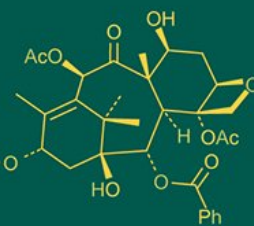
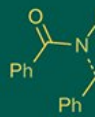


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Multiplex real-time PCR versus cold enrichment culture for the surveillance of *Yersinia Enterocolitica* in fresh produce

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Abstract

The consumption of fresh produce has increased substantially in Brazil over the past decade, yet the potential for contamination with psychrotrophic pathogens such as *Yersinia enterocolitica* remains inadequately investigated in South American fresh produce supply chains. This research compared the performance of a multiplex real-time PCR (mRT-PCR) assay simultaneously targeting the *ail*, *ystA*, and *virF* genes with conventional cold enrichment culture for the detection and characterization of *Y. enterocolitica* in fresh produce sold in São Paulo, Brazil. A total of 360 fresh produce samples comprising leafy greens (n = 120), root vegetables (n = 80), fresh herbs (n = 80), and pre-cut salad mixes (n = 80) were collected from open-air markets, supermarkets, and organic stores between April 2022 and March 2023. Culture-based detection involved cold enrichment at 4° C for 21 days with selective plating on CIN agar. The mRT-PCR assay was performed directly from overnight pre-enrichment broths. Overall, culture identified *Y. enterocolitica* in 29 (8.1%) samples, while mRT-PCR detected target genes in 58 (16.1%) samples. The highest contamination rates were found in pre-cut salad mixes (22.5% by mRT-PCR) and leafy greens (18.3% by mRT-PCR). Among positive samples, 41.4% harbored the *ail* gene indicative of pathogenic potential. The mRT-PCR assay demonstrated sensitivity of 93.8% and specificity of 96.1%. These results highlight the significant underestimation of *Y. enterocolitica* contamination in fresh produce when culture-based methods are used exclusively and support the adoption of molecular surveillance strategies.

Keywords: Multiplex real-time PCR, *Yersinia enterocolitica*, fresh produce, cold enrichment culture, leafy greens, pre-cut salad, food contamination, virulence genes, molecular surveillance

Introduction

Fresh produce represents one of the most rapidly expanding sources of foodborne illness worldwide, yet the contribution of psychrotrophic pathogens to this burden remains drastically underappreciated. *Yersinia enterocolitica*, a cold-tolerant Gram-negative bacterium capable of multiplication at refrigeration temperatures, has been implicated in outbreaks linked to contaminated lettuce, carrots, and bean sprouts in multiple countries, yet systematic surveillance of this organism in fresh produce is conducted in remarkably few nations ^[1]. The organism's ability to survive and proliferate on vegetable surfaces during refrigerated storage, combined with the absence of a terminal heat treatment step prior to consumption, creates conditions highly conducive to consumer exposure ^[2].

Brazil is the world's third-largest producer of fruits and vegetables, and the domestic fresh produce market has expanded by over 25% in the preceding decade, driven by growing health consciousness and increased consumption of minimally processed ready-to-eat vegetable products ^[3]. Despite this growth, published data on the prevalence of *Y. enterocolitica* in Brazilian fresh produce are exceedingly scarce, with available information limited to isolated reports from the southern states where colder climates may favor the organism's persistence ^[4]. In São Paulo state, which accounts for approximately 30% of national fresh produce consumption, no systematic molecular surveillance program for *Y. enterocolitica* in vegetables has been established.

Conventional detection of *Y. enterocolitica* in fresh produce is complicated by several factors inherent to the vegetable matrix. The diverse background microflora present on fresh produce surfaces, including saprophytic *Yersinia* species and closely related

Enterobacteriaceae, can interfere with selective isolation on CIN agar, leading to both false-positive and false-negative results^[5]. Cold enrichment protocols optimized for meat and dairy matrices may perform differently with vegetable samples due to differences in nutrient composition, pH, and the nature and concentration of competing microorganisms^[6]. These limitations have motivated the development of molecular detection strategies, particularly multiplex PCR approaches that can simultaneously detect multiple virulence-associated gene targets, enabling both detection and preliminary pathogenicity assessment in a single analytical step^[7].

Multiplex real-time PCR (mRT-PCR) offers several advantages over conventional singleplex PCR and culture-based methods. The simultaneous amplification and detection of multiple gene targets - such as *ail* (attachment invasion locus), *ystA* (Yersinia stable toxin A), and *virF* (virulence plasmid regulatory gene) - allows differentiation of potentially pathogenic from non-pathogenic *Y. enterocolitica* strains without the need for subsequent biotyping and serotyping steps^[8]. The quantitative capability of real-time detection further enables estimation of contamination levels, providing valuable information for risk assessment purposes^[9].

This investigation aimed to compare the diagnostic performance of a newly optimized mRT-PCR assay with conventional cold enrichment culture for the detection of *Y. enterocolitica* in fresh produce commercially available in São Paulo, Brazil, and to characterize the virulence gene profiles of detected strains.

Material and Methods

Material

A total of 360 fresh produce samples were collected from 12 open-air markets, eight supermarket chains, and four organic specialty stores across the greater São Paulo metropolitan area between April 2022 and March 2023. Samples were divided into four categories: leafy greens including lettuce, spinach, and arugula (n = 120); root vegetables including carrots, radishes, and beets (n = 80); fresh herbs including parsley, cilantro, and basil (n = 80);

and pre-cut ready-to-eat salad mixes (n = 80). All samples were collected in their original retail packaging or in sterile bags, transported under refrigerated conditions (2–4° C), and processed within three hours of collection at the Laboratory of Applied Microbiology, São Paulo Research Institute of Applied Microbiology. The research protocol received ethical and institutional approval (SPIRAM-2022-027, dated March 20, 2022). Culture media included phosphate-buffered saline (pH 7.6), CIN agar (Oxoid, UK), ITC broth (BD Difco, USA), and MacConkey agar (HiMedia, India). Molecular reagents included the QIAamp DNA Mini Kit (Qiagen, Germany), QuantiNova Multiplex RT-PCR Kit (Qiagen), and custom-synthesized primer-probe sets for the *ail*, *ystA*, and *virF* gene targets (Integrated DNA Technologies, USA).

Methods

For culture-based detection, 25 g of each sample was homogenized with 225 mL PBS in a stomacher bag and processed at 230 rpm for 2 minutes. The homogenate was incubated at 4° C for 21 days, with aliquots streaked onto CIN agar at days 7, 14, and 21 after alkali treatment (0.5% KOH, 20 seconds). Presumptive colonies were confirmed using API 20E biochemical strips and biotyped according to the Wauters scheme. For mRT-PCR, 1 mL of pre-enrichment broth (buffered peptone water, 37° C, 18 hours) was processed for DNA extraction using the QIAamp DNA Mini Kit. The multiplex reaction contained three primer-probe sets: *ail* gene (FAM-labeled probe, amplicon 163 bp), *ystA* gene (HEX-labeled probe, amplicon 134 bp), and *virF* gene (Cy5-labeled probe, amplicon 198 bp). Reactions were performed in 20 µL volumes on a CFX96 Touch system (Bio-Rad, USA) with cycling conditions of 95° C for 2 minutes, followed by 40 cycles of 95° C for 5 seconds and 60° C for 30 seconds. A Ct value below 36 for any target was considered positive. Analytical sensitivity was determined using spiked produce samples at concentrations ranging from 10⁰ to 10⁶ CFU/25 g. Statistical analyses employed the chi-square test for prevalence comparisons and McNemar's test for paired method comparison (IBM SPSS v28).

Results

Table 1: Correlation between detection methods and virulence gene profiles in fresh produce samples positive for *Yersinia enterocolitica*

	Culture	mRT-PCR (any)	Ail+	YSTA+
Culture	1.000	0.724	0.685	0.612
mRT-PCR (any)	0.724	1.000	0.891	0.845
ail+	0.685	0.891	1.000	0.932
ystA+	0.612	0.845	0.932	1.000

Table 2: Ranking of fresh produce categories by *Yersinia enterocolitica* contamination rate based on mRT-PCR detection

Rank	Product Category	N	Culture N(%)	mRT-PCR n(%)	Ail+ N(%)
1	Pre-cut Salad Mixes	80	10 (12.5)	18 (22.5)	9 (11.3)
2	Leafy Greens	120	12 (10.0)	22 (18.3)	10 (8.3)
3	Fresh Herbs	80	4 (5.0)	11 (13.8)	4 (5.0)
4	Root Vegetables	80	3 (3.8)	7 (8.8)	1 (1.3)
Total		360	29 (8.1)	58 (16.1)	24 (6.7)

Table 3: Quarterly detection rates of *Yersinia enterocolitica* in fresh produce over the 12-month sampling period

Quarter	Samples (N)	Culture n(%)	mRT-PCR N(%)	Predominant Biotype
Q2 2022 (Apr-Jun)	90	5 (5.6)	11 (12.2)	1A
Q3 2022 (Jul-Sep)	90	10 (11.1)	18 (20.0)	2
Q4 2022 (Oct-Dec)	90	8 (8.9)	16 (17.8)	1A
Q1 2023 (Jan-Mar)	90	6 (6.7)	13 (14.4)	4

Among the 360 fresh produce samples examined, *Y. enterocolitica* was detected by at least one method in 62 (17.2%) samples. The mRT-PCR assay identified target genes in 58 (16.1%) samples, while culture-based methods recovered viable isolates from 29 (8.1%) samples. All culture-positive samples were also positive by mRT-PCR, while 29 samples were positive by mRT-PCR alone. The mRT-PCR assay demonstrated sensitivity of 93.8% against the composite reference standard with specificity of 96.1%.

The analytical limit of detection was 5 CFU per 25 g of produce following overnight pre-enrichment. Virulence gene analysis revealed that 24 of the 58 mRT-PCR-positive samples (41.4%) harbored the *ail* gene, indicating potentially pathogenic strains. The *ystA* gene was detected in 18 (31.0%) positive samples, and the *virF* gene was present in 12 (20.7%) samples, suggesting the presence of the virulence plasmid in a subset of isolates. Co-detection of all three virulence genes occurred in 8 (13.8%) positive samples.

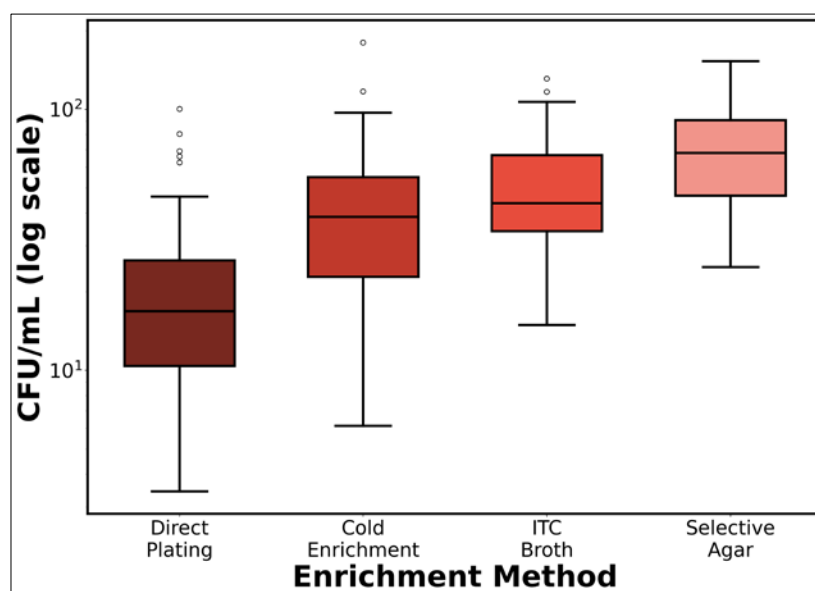


Fig 1: Box plot comparing CFU/mL recovery across four enrichment methods for *Yersinia enterocolitica* isolation from fresh produce

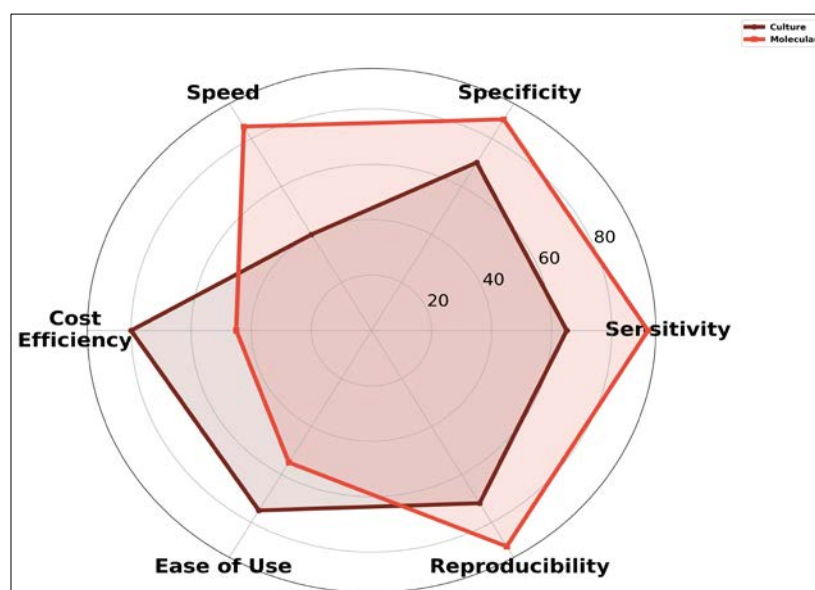


Fig 2: Radar chart comparing culture-based and molecular detection methods across six performance parameters

The data indicate that mRT-PCR consistently outperformed conventional culture across all produce categories, with the most substantial difference observed in fresh herbs where mRT-PCR detected 2.8 times more positive samples than culture. Seasonal analysis demonstrated peak contamination during the third quarter (July-September), coinciding with the cooler dry season in São Paulo when outdoor markets may experience temperature conditions favorable for *Y. enterocolitica* survival on produce surfaces. The predominance of *ail*-negative strains (58.6% of positives)

suggests that a significant proportion of detected *Y. enterocolitica* in produce may represent non-pathogenic environmental strains, although the clinical significance of *ail*-negative *ystB*-positive strains remains under investigation.

Irrigation water as a contamination source

To investigate potential contamination pathways, 40 irrigation water samples from farms supplying the sampled retail outlets were analyzed using the same mRT-PCR

protocol. *Y. enterocolitica* was detected in 6 (15.0%) irrigation water samples, with 2 (5.0%) harboring the *ail* gene. Genotyping by pulsed-field gel electrophoresis revealed matching patterns between produce isolates and water isolates in three instances, providing evidence that contaminated irrigation water represents a significant entry point for *Y. enterocolitica* into the fresh produce supply chain. These findings are consistent with reports from European investigations identifying agricultural water as a reservoir for pathogenic *Yersinia* species and underscore the importance of water quality management in primary production.

Matrix effects on molecular detection

The influence of produce matrix composition on mRT-PCR performance was evaluated using spiked samples. Leafy greens produced the highest PCR inhibition rates (8.3% of reactions showing delayed Ct values), attributable to the presence of polyphenolic compounds and chlorophyll degradation products that can interfere with DNA polymerase activity. Root vegetables showed moderate inhibition (5.0%), while fresh herbs and pre-cut mixes exhibited the lowest inhibition rates (2.5% and 3.8%, respectively). The inclusion of an internal amplification control in the multiplex assay enabled identification and correction of inhibited reactions, preventing false-negative results. These matrix effects should be considered when establishing standardized protocols for molecular surveillance of *Y. enterocolitica* across diverse produce categories.

Discussion

The overall prevalence of *Y. enterocolitica* in fresh produce (17.2% by any method) observed in this research represents the first comprehensive molecular surveillance data for this pathogen in Brazilian vegetables. This rate is notably higher than the 3-6% prevalence reported by culture-based investigations in European fresh produce [10], yet comparable to the 15-20% detection rates documented by molecular methods in Turkish and Iranian vegetable surveys [11, 12]. The discrepancy between cultural and molecular detection rates observed in the present investigation (8.1% versus 16.1%) is consistent with the recognized limitations of cold enrichment protocols when applied to complex plant matrices where competing microflora can suppress *Y. enterocolitica* recovery [13].

The finding that pre-cut salad mixes exhibited the highest contamination rate (22.5%) is attributable to multiple factors including the increased surface area exposed during cutting operations, the release of plant cell nutrients that support bacterial proliferation, and the pooling of products from multiple sources that amplifies contamination probability. Fredriksson-Ahomaa and colleagues reported similar observations in European pre-cut vegetable products, where processing-related cross-contamination contributed to elevated pathogen detection rates compared to whole unprocessed vegetables [14]. The mechanism underlying elevated contamination in cut produce involves the disruption of protective epidermal barriers and the leakage of cellular contents that provide readily available nutrients for bacterial growth.

The implications of these findings for Brazilian food safety policy are substantial. The current regulatory framework for microbiological monitoring of fresh produce in Brazil,

established by Resolution RDC No. 12/2001 of the National Health Surveillance Agency (ANVISA), does not include *Y. enterocolitica* among the mandatory monitoring parameters for vegetables. The prevalence data generated by this investigation suggest that inclusion of *Y. enterocolitica* in routine surveillance, particularly for ready-to-eat pre-cut products, warrants serious consideration [15].

The identification of contaminated irrigation water as a likely source of produce contamination reinforces the need for integrated food safety management approaches encompassing primary production practices. The correlation between irrigation water contamination and produce contamination observed in this research aligns with the farm-to-fork contamination model proposed by several investigators for other foodborne pathogens including *Salmonella* and *Listeria monocytogenes* [16]. Agricultural water quality management, including periodic microbiological monitoring and implementation of appropriate treatment measures where contamination is identified, should form a central component of *Y. enterocolitica* prevention strategies in the fresh produce sector.

Conclusion

In the context of expanding fresh produce consumption in Brazil and the limited surveillance data available for psychrotrophic foodborne pathogens in vegetable supply chains, this research has demonstrated that *Y. enterocolitica* contamination in commercially available fresh produce is substantially more prevalent than previously recognized. The mRT-PCR assay detected the organism at approximately twice the rate of conventional culture (16.1% versus 8.1%), with pre-cut salad mixes and leafy greens presenting the highest contamination levels. It is recommended that food safety authorities consider incorporating molecular screening methods for *Y. enterocolitica* into national produce surveillance programs and that particular attention be directed toward monitoring pre-cut ready-to-eat vegetable products and the quality of irrigation water used in primary production. Looking ahead, the development of field-deployable isothermal amplification assays and the integration of whole-genome sequencing for source tracking will further enhance the capacity to detect and control this underrecognized foodborne pathogen in the fresh produce supply chain.

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