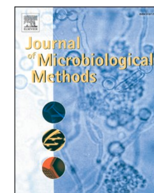
Journal of Microbiological Methods 236 (2025) 107207

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth



Validation of a Relative Centrifugal Force method for the enumeration and detection of *Campylobacter* from chicken carcass rinsates

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ARTICLE INFO

Keywords:
Enumeration method
Centrifugation
Poultry meat
Hygiene indicator
Regulatory testing

ABSTRACT

Campylobacteriosis is the most frequently notified foodborne disease in New Zealand and poultry is the predominant infection source. New Zealand monitors *Campylobacter* present in poultry carcass rinsates under the National Microbiological Database (NMD) programme. To better monitor *Campylobacter* control improvements, a more sensitive method is required that can enumerate rinsates with lower *Campylobacter* numbers. This study developed a modification of the current NMD method involving adding a relative centrifugal force (RCF) step for concentrating *Campylobacter* from poultry carcass rinsates. Centrifugation for 30 min significantly improved *Campylobacter* recovery compared with 15 min ($p < 0.001$), but there were no differences between RCFs of 3500, 4000 and 4430 x g ($p = 0.992$). RCF and NMD method performances were compared in a single laboratory validation study that used different inoculation levels of twelve *Campylobacter* strains, including poultry isolates. *Campylobacter* was detected from more samples ($p < 0.001$) using the RCF method (93 of 126; 73.8 %) than the NMD method (65 of 126; 51.6 %). The RCF method had a seven-fold lower detection limit (28 colony forming units (CFU)/400 ml) than the NMD method (200 CFU/400 ml). The detection limit accounted for an observed 70.3 % of the inoculated CFU captured within the centrifuged pellet. *Campylobacter* was also detected from significantly more ($p < 0.001$) commercial chicken rinsate samples tested by poultry industry laboratories using the RCF method (257 of 863; 29.8 %) than the NMD method (114 of 863; 13.2 %). Taken together, results support the RCF method as a modification of the NMD method to enumerate lower numbers of *Campylobacter* in rinsates.

1. Introduction

Campylobacteriosis, primarily caused by the species *Campylobacter* (*C.*) *jejuni* and *C. coli,* is the most frequently notified foodborne disease in New Zealand (Armstrong et al., 2024). An approximately 50 % reduction of human cases of campylobacteriosis was observed in New Zealand following implementation of poultry chain-focused measures during 2006–2008 (Sears et al., 2011). Since that time there has been only a gradual reduction in incidence whereas a substantial reduction is required (Armstrong et al., 2024). Further reduction of human cases of foodborne campylobacteriosis is a strategic priority for New Zealand Food Safety (NZFS). During 2020, NZFS published a *Campylobacter* Action Plan which introduced a goal of reducing the incidence of domestically acquired, foodborne campylobacteriosis by 20 % from 88 to 70

Abbreviations: National Microbiological Database, NMD; relative centrifugal force, RCF; New Zealand Food Safety, NZFS; colony forming units, CFU; Buffered Peptone Water, BPW; modified Charcoal Cefoperazone Deoxycholate Agar, mCCDA; positive agreement, PA; negative agreement, NA; positive deviation, PD; negative deviation, ND; limit of detection (LOD_{50}) and 90%, LOD; relative limit of detection, RLOD.

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https://doi.org/10.1016/j.mimet.2025.107207
Received 8 May 2025; Received in revised form 29 July 2025; Accepted 29 July 2025
Available online 31 July 2025
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per 100,000 population by the end of 2024 (New Zealand Food Safety, 2023). Poultry remains the most important vehicle for human infection in New Zealand, with a recent study estimating that 84 % of campylobacteriosis cases could be assigned to a poultry source (Lake et al., 2021). Accordingly, the *Campylobacter* Action Plan has a primary focus on the poultry food supply chain.

Since 2007, a bacteriological spread plate method has been used as part of the National Microbiological Database (NMD) programme to enumerate *Campylobacter* from rinsates collected from poultry carcasses following primary processing (Ministry for Primary Industries, 2023). The method involves plating 2 ml over six plates of selective medium (modified Charcoal Cefoperazone Deoxycholate Agar; mCCDA) of each 400 ml rinsate (for broiler or end-of-lay chickens or ducks; detection limit of 2.39 log_{10} colony forming units (CFU)/carcass rinsate) or 600 ml rinsate (for turkeys or breeder chickens; detection limit of 2.48 log_{10} CFU/carcass rinsate). The NMD rinsate results are used to set performance targets for the processing plants and poultry producers. Ongoing improvement in the hygienic dressing of carcasses by the New Zealand poultry industry (Kingsbury et al., 2023; Lake et al., 2013; Sears et al., 2011) has resulted in a reduction in the numbers and prevalence of *Campylobacter* observed under the NMD programme. *Campylobacter* prevalence for meat chicken carcass rinsates decreased from 38.9 % in 2010 to 10.0 % in 2023, and prevalence of high-level positive samples (>3.78 log_{10} CFU/400 ml carcass rinsate) decreased from 6.1 % in 2010 to 1.1 % in 2023 (Ministry for Primary Industries data). There was a less dramatic reduction in average numbers per rinsate (2.39 log_{10} CFU in 2010 compared with 2.09 log_{10} CFU in 2023) because numbers were already close to the detection limit of the enumeration method. As such, it has become increasingly difficult to measure further microbiological improvements. Therefore, the development of a more sensitive method that can enumerate rinsates with lower numbers of *Campylobacter* than the current NMD method LOD would have benefit for the poultry industry and for regulatory purposes.

The new method proposed for this study was based on the addition of a concentration step to the current NMD method using centrifugation to improve the recovery rate of *Campylobacter* spp., hereafter referred to as the Relative Centrifugal Force (RCF) method. Because it is a modification of the NMD method, there are no changes to the plating medium, incubation or confirmation steps, so the evaluation of specificity is not required. The key components of the proposed method were initially developed and used for a research project that enumerated *Campylobacter* on carcasses through primary and secondary processing (Kingsbury et al., 2023). Centrifugation has been used for concentrating *Campylobacter* from dairy products prior to enrichment in reference methods (Hunt et al., 2001), and from poultry rinsates for enrichment and enumeration in published studies (Kingsbury et al., 2023; Mullner et al., 2009; Nannapaneni et al., 2005; Nohra et al., 2016; Tangvatcharin et al., 2005). However, centrifuged volumes and centrifugation parameters differed between studies, and data were not found that specifically assessed the efficacy and percentage recovery of *Campylobacter* following centrifugation from poultry rinsates, or how the method performs using different strains.

The objectives of this study were:

1. Method optimization: Test and confirm the most effective RCF and centrifugation time for maximum *Campylobacter* recovery, using parameters that are attainable by poultry carcass rinsate-testing laboratories in the New Zealand Recognised Laboratory Programme.
2. Single laboratory validation: Use the parameters defined by the method optimization to validate the RCF (modified) method for the detection and enumeration of *Campylobacter* from artificially inoculated chicken carcass rinsates against the current NMD programme (reference) method using a single laboratory validation.
3. Commercial laboratory testing: Test commercial chicken carcass rinsates using the RCF (modified) method alongside the NMD programme testing by Recognised Laboratory Programme poultry industry laboratories.

2. Materials and methods

2.1. Source and testing of broiler chicken carcass rinsates

The matrix for the experimental testing included broiler chicken (produced primarily for meat) carcass rinsates to ensure that experiments were conducted as close as possible to real conditions. Chicken carcass rinsates may have a high level of fat, proteins, other microbes and inhibitors, which are not found in water, buffer, or enrichment broth, and may influence the quantification of *Campylobacter*.

Rinsates were obtained from Christchurch, New Zealand-based poultry processors during early spring and arose from *Campylobacter*-negative (as determined from testing of caecal samples prior to harvesting), barn-raised, first-cut flocks, which increased the likelihood that there will be no background *Campylobacter* present in the collected rinsates. The rinsates were collected from carcasses at the end of primary processing (where NMD samples are also collected) by trained NMD programme samplers. Briefly, 400 ml Buffered Peptone Water (BPW) with sodium thiosulphate (0.003 % (weight by volume); Fort Richard Laboratories, Auckland, New Zealand) was poured onto the carcass in the rinsate bag and the carcass was rinsed for two minutes. Following the rinse procedure, the corner of the sample bag was aseptically snipped and the entire volume of rinsate was collected back into the relabelled bottle which originally contained the BPW for the sample. Rinsates were delivered in cooler boxes with ice packs to the testing laboratory within two hours. At the laboratory, rinsates were pooled into batches relevant to the volumes required per experiment; batches of 1625 ml volumes for method optimization and 2825 ml volumes for validation testing. Rinsate batches for validation testing were then aliquoted into seven 400 ml volumes in bottles, each labelled with the batch number. Rinsates were frozen and stored at −20 °C until required. Although freezing may kill some of the background flora, this was the only practical option. Rinsates were placed at 4 °C to thaw 4–5 days prior to inoculation.

Before freezing, aliquots of each batch were spread-plated onto mCCDA plates (0.33 ml per plate, six plates per batch), and incubated for 48 ± 2 h at 42 ± 0.5 °C. Plates were observed for the presence of suspect *Campylobacter* colonies (typical colonies are greyish, often with a metallic sheen, and are flat and moist with a tendency to spread); all samples tested negative for *Campylobacter*, and non-*Campylobacter* bacterial colonies were also not observed. While background *Campylobacter* was below the level of detection by direct plating (<1 CFU in 2 ml for each batch tested), it remained possible that lower concentrations or stressed *Campylobacter* were present in initial rinsates. Note that although initial rinsates were not tested by enrichment culture, *Campylobacter* was not detected from uninoculated rinsates by enrichment or by direct plating during the single laboratory comparison experiments (Section 3.2).

All media and diluents used in the study were sourced from Fort Richard Laboratories, Auckland, New Zealand. All *Campylobacter* culturing was carried out under a microaerobic atmosphere provided by the use of CampyGen™ gas sachets (2.5–9.5 % CO_2, 6.2–13.2 % O_2; Oxoid, ThermoFisher Waltham, MA)) in an airtight container. The mCCDA plates were dried before use (up to 8 h at 37 °C with minimal light exposure) which was critical for allowing higher volumes (0.33 ml) of plated rinsate to be absorbed and for preventing spreading or swarming growth of colonies.

2.2. Strain selection

Because both the reference and the RCF method use the same selective medium, a full panel of inclusivity and exclusivity strains was not required for the study for measuring method specificity. However, it was

important to demonstrate that the RCF method performed similarly for a range of *Campylobacter* strains in the event that some strains are more sensitive to centrifugation. Strains used in the study are listed in Table 1, and the rationale for their selection was guided by ISO 16140-2:2016 recommendations (International Organization for Standardization, 2016). All strains had been categorized at least to the stage of species, and the majority had been whole genome sequenced and/or had the sequence type (ST) determined. Strains were selected to cover a range in phenotypic diversity. The three *Campylobacter* species most commonly isolated from poultry were included; *C. jejuni*, *C. coli*, and *C. lari*, with more *C. jejuni* strains tested because this is the most common species isolated from poultry in New Zealand. Reference type strains were included to allow equivalent studies to be conducted internationally; all other strains were New Zealand poultry isolates. Sequence types (STs) that belong to different clonal complexes were selected based on the most common STs identified from New Zealand poultry in recent studies (Kingsbury et al., 2023; Lake et al., 2021). Strains from both the North Island and South Island of New Zealand were included.

Table 1
Bacterial strains used in this study.

Species	Strain name/s	Description, source[1]	Reference[2]
Campylobacter (C.) jejuni	ATCC 33560, NZRM 2397	ST403. Isolated from bovine faeces. Type strain, laboratory reference strain.	
	P1566e	ST6964. Isolated from retail chicken; North Island, NZ.	(French et al., 2019)
	CMB09552	ST45. Isolated from retail chicken; South Island, NZ.	(Gilpin et al., 2013)
	CMB090876	ST48. Isolated from retail chicken liver; South Island, NZ.	(Gilpin et al., 2013)
	P1539a	ST50. Isolated from chicken carcass during processing; North Island, NZ.	
	PL0183	ST2927. Isolated from chicken carcass during processing; North Island, NZ.	(Kingsbury et al., 2023)
C. coli	ATCC 33559, NZRM 2607	ST900. Isolated from pig faeces. Type strain, laboratory reference strain.	
	P1572b	ST2256. Isolated from chicken carcass; North Island, NZ.	
	CMB09566	ST1581. Isolated from retail chicken liver; South Island, NZ.	(Gilpin et al., 2013)
C. lari	ATCC 35221, NZRM 2622	Isolated from herring gull cloacal swab. Type strain, laboratory reference strain.	
	PL0385	Isolated from chicken carcass during processing; North Island, NZ.	(Kingsbury et al., 2023)
	CMB91863	Isolated from retail chicken; South Island, NZ.	(Gilpin et al., 2013)
Escherichia coli	ATCC 25922, NZRM 916	Laboratory reference strain. Negative control for mCCDA plating.	
Pseudomonas aeruginosa	ATCC 25668, NZRM 981	Laboratory reference strain. Positive control for APC.	

[1] Abbreviations: NZ, New Zealand; APC Aerobic Plate Count.
[2] Cultures were obtained from the New Zealand Institute for Public Health and Forensic Science's (PHF Science) New Zealand Reference Culture Collection, PHF Science's Christchurch Science Centre culture collection, and from [m]Epi-Lab, Massey University.

2.3. Inoculum preparation and inoculation of rinsates

Strains were revived from frozen stocks by plating onto Columbia Sheep Blood Agar (CBA), and incubated at 42 °C for 48 h in a sealed container under microaerobic atmosphere. Strains were then cultured and maintained in Preston broth for no more than one week per subculture. Approximately 96 h before the inoculum preparation, the strains were streaked to mCCDA plates as well as CBA plates to check for purity. The mCCDA plates were used to inoculate fresh mCCDA plates 48 h before inoculum preparation.

A cold-preconditioned inoculum was prepared 48 h prior to each rinsate inoculation, as was used and described in previous studies (Kingsbury and Soboleva, 2021; Rivas et al., 2021). Preconditioned inocula are often recommended to adapt the culture to specific conditions that may be applicable to the specific characteristics of the matrix being tested (National Advisory Committee On Microbiological Criteria For Foods, 2010). Specifically, a suspension of the strain was prepared in 5 ml Dulbecco's Phosphate Buffered Saline (1×) without calcium or magnesium (PBS) by swabbing colonies from a freshly cultured mCCDA plate. The optical density at 600 nm (OD_{600}) of the PBS/*Campylobacter* suspension was adjusted to approximately 0.3 (corresponding to $\sim 10^8$ CFU/ml). The OD_{600} value provided only a rough approximation of CFU; thus, plating was required to get the best estimate of viable CFU present. A 10-fold serial dilution series was prepared in PBS. On the day of inoculum preparation, the highest dilutions (10^{-5} and 10^{-6} tubes containing $\sim 10^3$ and $\sim 10^2$ CFU per ml, respectively) were plated to mCCDA (selective medium for *Campylobacter*) and CBA plates (to verify that there was no contamination present); 6 × 0.33 ml per plate per dilution). Serial dilutions were stored in PBS for 48 h at 4 °C in an airtight container under microaerobic conditions until rinsate inoculation. Colony counts from the plating of the inoculum on mCCDA on the day of inoculum preparation were used to calculate the actual numbers present in the *Campylobacter* inoculum suspension, and the volume of that inoculum suspension that would need to be added to rinsates to achieve the desired CFU per ml. On the day of rinsate inoculation (48 h after inoculum preparation), the *Campylobacter* inoculum was again plated to estimate the actual CFU inoculated. Because some reduction in *Campylobacter* viability occurs during storage, pilot experiments were first undertaken for each strain to calculate the average recovery rate in CFU after 48 h storage; pilot reduction rates ranged from 0.3 to 0.8 depending on the strain. The recovery rates were taken into account when calculating inoculum volumes added to rinsates.

Innocula were added to chilled chicken carcass rinsates. Inoculated rinsates were swirled and gently inverted five times (but not shaken) to disperse the inoculum within the sample. Inoculated rinsates for the method optimization experiments were stored under refrigeration and tested on the following day. This mimics what may occur during testing of poultry rinsate samples by the NMD programme method whereby samples may be tested on the day following sampling, and must be tested no longer than 30 h after sample collection (Ministry for Primary Industries, 2023). To provide the best estimates for CFU inoculated, rinsates for the single laboratory validation experiments were inoculated approximately 0.5–2 h prior to experimentation.

2.4. Method optimization: refining the most effective Relative Centrifugal Force (RCF) and time

Centrifugation parameters were guided by the maximum RCF of centrifuges from commercial laboratories that conduct NMD programme testing of poultry rinsates. Three different RCF (x g) were tested to ascertain the most effective RCF or range of RCFs; 3500, 4000 and 4430 x g. A further variable tested was centrifugation time; methods used in a recent study used 30 min centrifugation (Kingsbury et al., 2023), while a shorter time of 15 min would reduce testing time in laboratories and would reduce potential heating of samples under non-refrigerated centrifugation conditions.

Specific methods for the method optimization experiments were conducted as depicted in Fig. 1A. Chicken carcass rinsates were inoculated the day prior to experimentation with a high inoculum level (target of 7.3 × 10^4 CFU/1625 ml; 45 CFU/ml), followed by storage overnight in the refrigerator. This concentration was deemed sufficient to provide comparisons to be made between non-centrifuged (NMD reference method) and centrifuged (RCF modified method) samples. The *Campylobacter* type and reference strains *C. jejuni* ATCC 33560 and *C. coli* ATCC 33559 (Table 1) were used for method optimization; testing for each strain was conducted on separate days.

The day following rinsate inoculation (after approximately 18–20 h), the rinsate was again swirled and gently inverted five times, to achieve a homogeneous sample. Volumes of 50 ml were dispensed into 31 labelled 50 ml sterile centrifuge tubes using a 50 ml sterile serological pipette. Five technical replicates for each strain were tested for each different RCF variable. Centrifugation was conducted with the centrifuge set to 18 °C because some Recognised Laboratory Programme laboratories do not have access to refrigerated centrifuges. The temperature of a subset of tubes before and after centrifugation was tested using an infrared thermometer (Novatech, United States) to ascertain the degree of temperature increase of samples; temperatures approximated 18 °C, the temperature of the centrifuge. Following centrifugation, a 45 ml volume of supernatant was pipetted off into a labelled tube using a 50 ml sterile serological pipette, leaving the remaining supernatant that was close to the pellet (the remaining supernatant plus pellet totalling 5 ml). The pellet was resuspended in the remaining 5 ml of supernatant using a vortex mixer on high for ~20 s. Note that although vortexing would incorporate more oxygen into samples, samples were plated to mCCDA (which contains oxygen-quenching agents) immediately following resuspension, and pilot experiments had determined that ~20 s vortexing resulted in higher CFU than by resuspending the pellet by pipetting up and down. Samples were spread-plated onto mCCDA plates. For uncentrifuged samples (reference method), six plates were inoculated with 0.33 ml each. For the centrifuged samples of resuspended

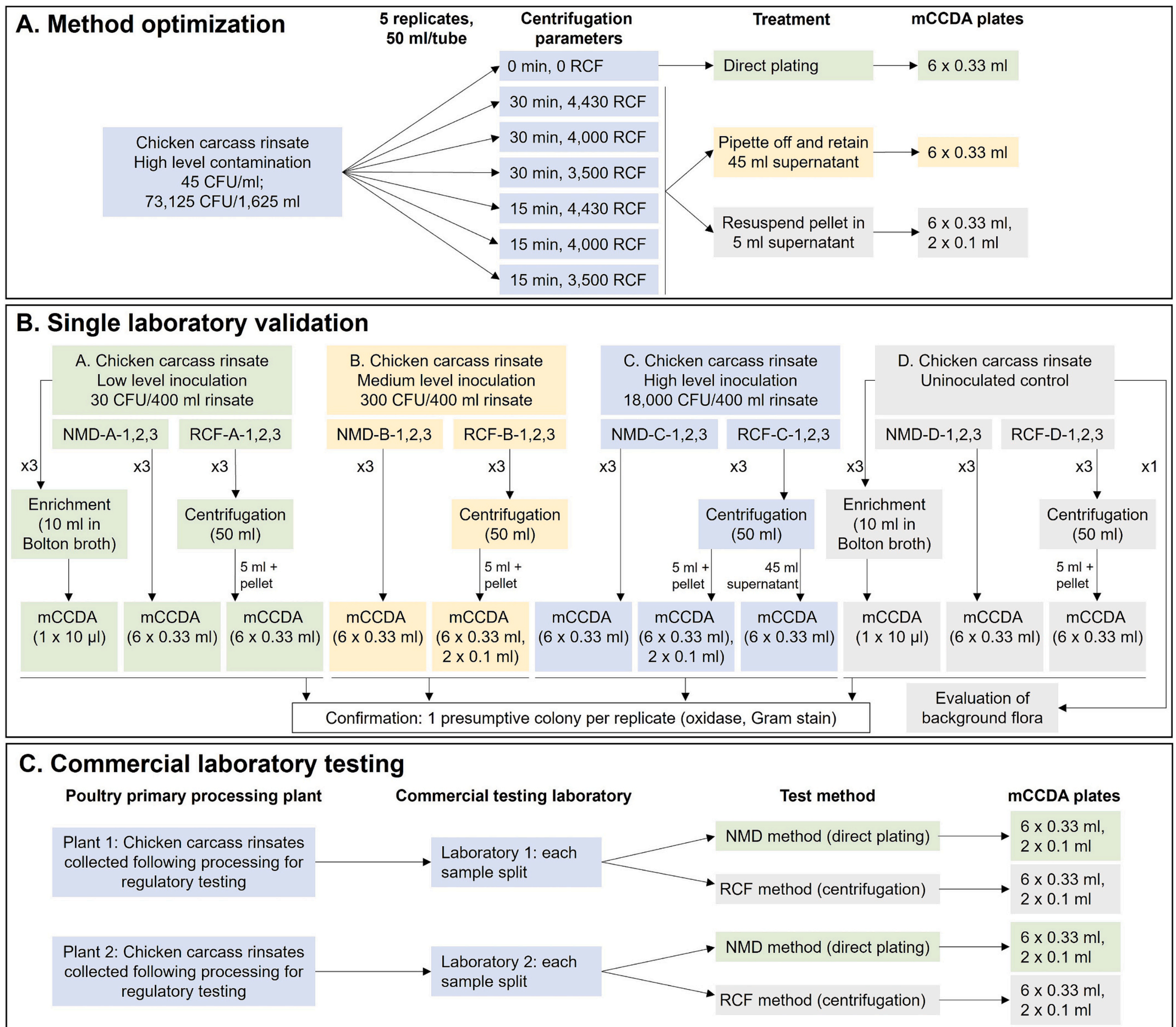

Fig. 1. Overview of methodology for (A) Method optimization to refine the most effective Relative Centrifugal Force (RCF) parameters using reference strains of *Campylobacter*; (B) Single laboratory validation comparing the RCF (modified) method with the NMD programme (reference) method using reference and poultry isolates of *Campylobacter*, and (C) Commercial laboratory testing involving a method comparison using naturally contaminated chicken carcass rinsates collected for regulatory purposes and tested by commercial poultry testing laboratories.

pellet, six plates were inoculated with 0.33 ml of resuspended pellet and a further two plates with 0.1 ml each. Aliquots of 0.33 ml from the 45 ml of discarded supernatant from each sample were inoculated onto each of six plates, to ascertain how many CFU were lost in the discarded supernatant.

2.5. Single laboratory validation: comparison of the NMD (reference) and RCF (modified) method for enumerating and detecting Campylobacter

Methodology for comparing the NMD (reference) method with the RCF (modified) method for enumerating and detecting *Campylobacter* from chicken carcass rinsates, was conducted as depicted in Fig. 1B. Experiments tested twelve different strains including six *C. jejuni*, three *C. coli,* and three *C. lari*, including the reference strains for each species and New Zealand poultry isolates (Table 1). Two strains were tested per experimental setup.

Each strain was inoculated at four target *Campylobacter* levels to span the analytical range of the test methods; low, medium, and high inoculum levels and an uninoculated control, as specified by ISO 16140-2:2016 Microbiology of the food chain - Method validation Part 2: Protocol for the validation of alternative (proprietary) methods against a reference method (International Organization for Standardization, 2016):

A. Low level target: 30 CFU/400 ml rinsate (i.e. 0.075 CFU/ml), which was modestly higher than the theoretical detection limit of the RCF method (defined as the lowest concentration of an analyte that can be detected by using a given analytical procedure; i.e. 20 CFU/400 ml rinsate based on 100 % recovery of *Campylobacter* in the centrifuged pellet). The level approximates a detection limit of 29 CFU/400 ml rinsate observed in an earlier pilot study.
B. Medium level target: 300 CFU/400 ml rinsate (i.e. 0.75 CFU/ml), which is modestly higher than the detection limit of the reference NMD method (200 CFU/400 ml rinsate).
C. High level target: 18,000 CFU/400 ml rinsate (i.e. 45 CFU/ml), which would allow 15 colonies per plate for direct plating (as per the NMD method).
D. Control: no added *Campylobacter* (i.e. 0 CFU/ml).

For single laboratory validation testing of samples using the NMD method, 0.33 ml was spread-plated onto each of 6 mCCDA plates (2 ml over 6 plates). For testing samples using the RCF (modified) method, three 50 ml volumes of each *Campylobacter* level were dispensed into labelled 50 ml sterile centrifuge tubes using 50 ml sterile serological pipettes. The tubes were centrifuged at 18 °C at 4430 x*g* for 30 min (as defined in method optimization experiments). Following centrifugation, a 45 ml volume of supernatant was pipetted off with a 50 ml sterile serological pipette, which was retained from centrifuged high level-inoculated samples, and discarded for other samples. The retained supernatant was spread-plated onto mCCDA (6 × 0.33 ml). The remaining 5 ml of supernatant and pellet were resuspended by vortexing for 20 s. For each replicate, 6 × 0.33 ml (for all analyte levels) and 2 × 0.1 ml (for 300 CFU and 18,000 CFU/400 ml target inoculation level samples to be used in the event that colonies were too numerous to count from the 0.33 ml plates) were spread-plated onto mCCDA plates. All mCCDA plates were incubated in a microaerobic atmosphere at 42 ± 0.5 °C for 48 ± 2 h. After this time, presumptive *Campylobacter* colonies were counted and recorded. The allowable counts for mCCDA direct plating of *Campylobacter* is 0–150 CFU/plate as per the NMD method (Ministry for Primary Industries, 2023); when counts exceeded this for plating of centrifuged samples, counts from the plating of 0.2 ml (0.1 ml per plate) were instead used and multiplied by a factor of 10.

Rinsates with low level contamination and uninoculated control rinsates (Samples A and D, Fig. 1B) were also tested for *Campylobacter* following enrichment (International Organization for Standardization, 2017). Three 10 ml volumes from each sample were individually added to Bolton broth (90 ml). Enrichments were incubated for 4 h at 37 °C followed by 44 h at 41.5 ± 0.5 °C in a microaerobic atmosphere. Following enrichment, 10 μl volumes of each enrichment were plated by streaking for single colonies onto mCCDA medium.

2.6. Confirmation of Campylobacter

One suspect colony per sample replicate tested underwent confirmation tests. Tests included detection of oxidase activity (Microbact oxidase strips, Oxoid, Hampshire, UK) and Gram stain, as per the NMD method.

2.7. Enumeration of background flora

For each batch of uninoculated rinsate for the single laboratory validation, the background flora were enumerated by Aerobic Plate Count (APC) according to the APHA Mesophilic aerobic plate count method (Ryser and Schuman, 2016). This was undertaken on the same day that each validation experiment was carried out.

2.8. Statistical analyses of data

The colony count data from method optimization experiments were analyzed to determine the most effective centrifugation RCF and time to be used in subsequent experiments. Data analyses were performed using R (R Core Team, 2023). Colony counts obtained from the three centrifuge RCF and two centrifuge times were first compared using an ANOVA test to demonstrate whether any sets of data differed, followed by a Tukey's Honest Significant Difference ad hoc test for pairwise comparisons between the different parameters (Yandell, 1997). Colony count data from the plated discarded supernatants were analyzed using the same methods to determine if there were significant differences between the methods. A further assessment looked at the homogeneity of data variances for the replicates within each parameter using Levene's and Fligner-Killeen tests (Conover et al., 1981; Fox, 2015). The most effective RCF conditions were deemed as those that had the best recovery of viable *Campylobacter* in the cell pellet, which would presumably correlate with the lowest viable *Campylobacter* present in the discarded supernatant. Ideally, the most effective method would also be the most consistent (less deviation in counts between replicates).

For the single laboratory validation, the performance parameters for the calculation and interpretation of sensitivity and the relative limit of detection (RLOD) of the methods were calculated for each strain tested, as defined by ISO 16140-2:2016 (International Organization for Standardization, 2016). The performance parameters informed whether the RCF (modified) method was more sensitive and could enumerate chicken carcass rinsates with numbers of *Campylobacter* lower than the current LOD under New Zealand Recognised Laboratory Programme laboratory conditions, for a genotypically diverse selection of New Zealand poultry isolates of *Campylobacter.* Performance parameters included:

- Positive agreement (PA): number of samples positive by both methods
- Negative Agreement (NA): number of samples negative by both methods
- Positive deviation (PD): number of samples negative by the NMD (reference) method but positive by RCF (modified) method
- Negative deviation (ND): number of samples positive by the NMD (reference) method but negative by RCF (modified) method
- Sensitivity of the NMD (reference) method (SE_{ref}): (PA + ND)/(PA + ND + PD) x 100
- Sensitivity of RCF (modified) method (SE_{mod}): (PA + PD)/(PA + ND + PD) x 100
- The limit of detection of each method at 50 % (LOD_{50}) and 90 % (LOD_{90}) confidence intervals, defined as the level of contamination

with an expectation of 50 % or 90 % positive test results for the method. These were calculated using the PODLOD calculation program version 9 Excel spreadsheet (Wilrich and Wilrich, 2009).

- Relative limit of detection (RLOD): the ratio of the LOD of the RCF (modified) method to that of the NMD (reference) method.

To compare sample prevalence between test methods, two-sample tests for equality of proportions with continuity correction were conducted using the 'prop.test' function in R (R Core Team, 2023).

2.9. Method comparison using commercial samples

Two poultry industry laboratories tested commercial chicken carcass rinsates received over a six-month period (19 April to October 2021) by both the NMD (reference) and RCF (modified) methods (Fig. 1C). This included a pilot study with 60 rinsates at one laboratory, which differed slightly whereby the supernatant was poured off instead of being pipetted off and a filter bag was used. As per the NMD (reference) method, a sample was reported positive if at least one colony was detected and confirmed on any of the six mCCDA plates for the respective method.

3. Results and discussion

3.1. Refining the most effective Relative Centrifugal Force (RCF) and time

The optimal methodology options for the RCF protocol were defined as those that gave the highest colony counts (best recovery) and best consistency. Variables tested included three centrifugation RCFs (3,500, 4,000 and 4,430 xg) and two centrifugation times (15 and 30 min). The suite of variables was tested for two reference strains (*C. jejuni* ATCC 33560 and *C. coli* ATCC 33559). A summary of results for the evaluation of methodology options for the centrifugation protocol are displayed in Fig. 2, and the data are provided in Supplementary Table S1. While both datasets were considered for evaluating the most effective centrifugation conditions, CFU data for *C. coli* ATCC 33559 were substantially lower than target levels and the *C. jejuni* ATCC 33560 levels (due to a greater die-off in the chicken carcass rinsate matrix prior to testing). Therefore, greater weight was placed on the *C. jejuni* ATCC 33560 data.

First, homogeneity of data variance was assessed as a measure of the consistency of each centrifugation treatment. ANOVA also assumes homogeneity of variance, which means that the variance among the groups should be approximately equal. There were no significant differences in variance for the different treatments and for either strain, as assessed

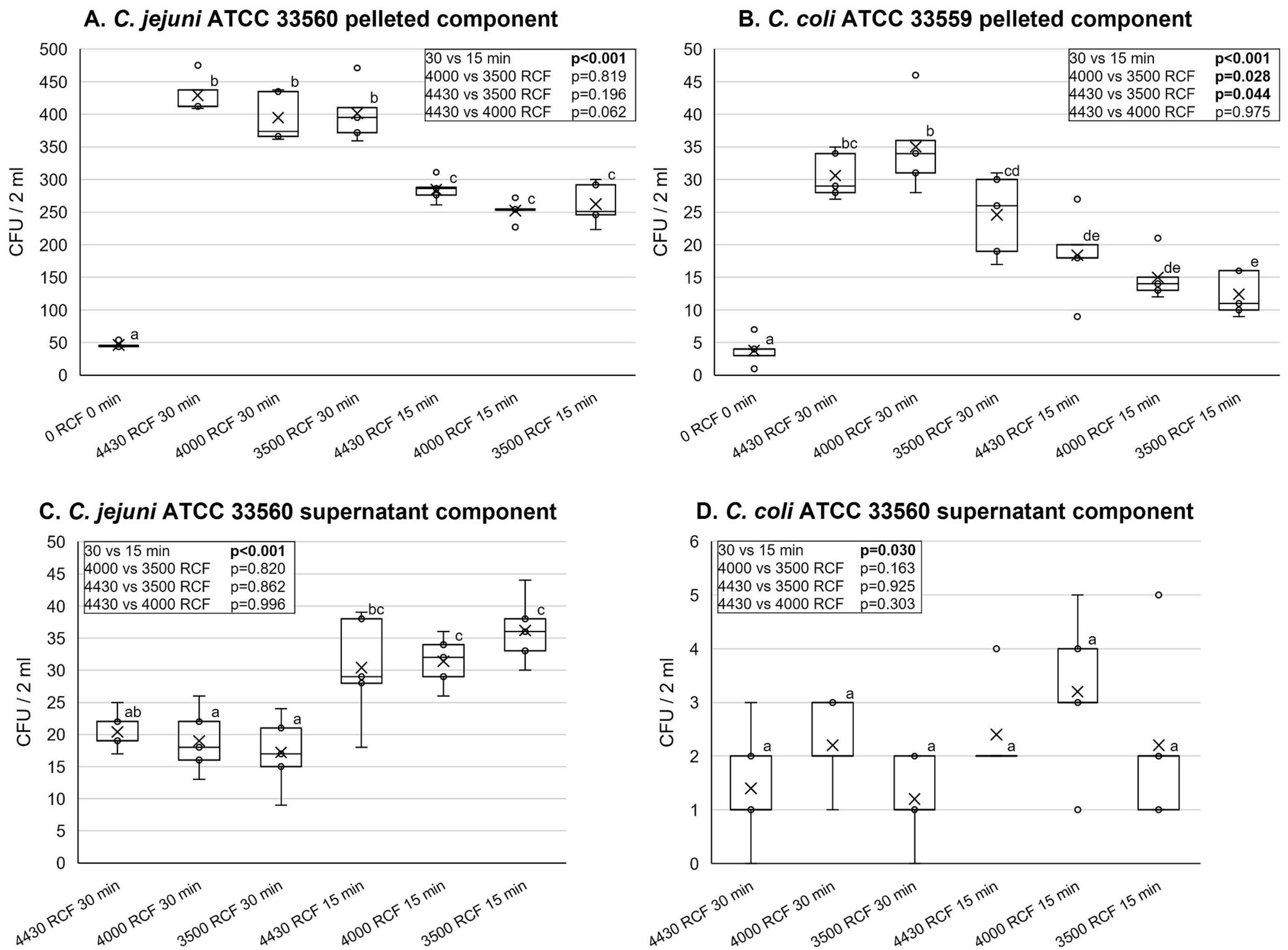

Fig. 2. Box and whisker plots of *Campylobacter* colony counts for 2 ml of pelleted component (A, B) and supernatant component (C, D) using different relative centrifugal force (RCF) and time (min). Colony counts from 2 ml uncentrifuged samples (direct plating; 0 RCF, 0 min) is also provided as a comparison (A, B). The solid lines within the boxes mark the median of five replicates, the X marks the mean, the bottom and top box borders represent the 25th and 75th percentiles, and whiskers above and below the box indicate the maximum and minimum values when no outliers were present. The *p*-values for centrifugation time and RCF comparisons are shown in the tables; bolded text indicates that p-values are significant ($p < 0.05$). Statistical differences for pairwise comparisons are indicated by different letters above plots; statistics are also provided in Table S4.

using Levene's and Fligner-Killeen tests of variance (Supplementary Table S2).

The mean CFU/2 ml obtained for plating of the pelleted fraction for each centrifugation variable was notably higher than obtained from direct plating (0 RCF, 0 min; Fig. 2A,B). The highest mean number of colonies obtained from the pelleted component for strain *C. jejuni* ATCC 33560 was using a centrifugation duration of 30 min and 4,430 RCF (429.0 CFU/2 ml; Fig. 2, Supplementary Tables S1 and S3, Supplementary Fig. S1). The lowest mean number of colonies was from 4,000 RCF for 15 min. The lowest count variance (259.7) was from 15 min centrifuge duration at 4,000 RCF. For *C. coli* ATCC 33559, the highest mean counts were obtained using 4,000 RCF for 30 min, and the lowest using 3,500 RCF for 15 min which also had the lowest variance.

When *C. jejuni* NZRM 2397 colony count data for all centrifugation RCFs were combined, colony counts from the pelleted components were higher following 30 min centrifugation than 15 min, and these differences were significant by ANOVA analysis ($p < 0.001$; 1 degree of freedom). However, there were no significant differences between centrifugation RCFs ($p = 0.063$; 2 degrees of freedom). There were significant differences ($p < 0.001$) between pairwise combinations of each RCF for 30 min compared with 15 min (Supplementary Table S4).

For *C. coli* NZRM 2607, ANOVA results also showed significant differences in recovery between centrifuge durations ($p < 0.001$; 1 degree of freedom), as well as RCF ($p = 0.018$; 2 degrees of freedom). As for *C. jejuni*, there were significant differences between most 30 min and RCF pairwise combinations compared with 15 min and RCF pairwise combinations (Supplementary Table S4). In addition, at a run time of 30 min, and 4,000 RCF resulted in significantly higher colony counts than 3,500 RCF, but there were no differences between colony counts at 4,430 and 4,000 RCF for a 30 min centrifugation.

The most effective centrifugation treatment may also have the fewest colony forming units discarded in the supernatant. For *C. jejuni* NZRM 2397, the lowest mean colony counts from the supernatant component were obtained following 30 min centrifugation at 3,500 RCF, and the highest were from 15 min centrifugation at 3,500 RCF (Fig. 2, Supplementary Tables S1 and S3, Supplementary Fig. S1). For *C. coli* NZRM 2607, the lowest counts were also from 30 min centrifugation at 3,500 RCF and highest from 15 min centrifugation at 4,000 RCF. Note however that colony counts were very low for the supernatant components for all centrifugation treatments for this strain because the inoculation level was lower. Although all attempts were made to inoculate the equivalent CFU levels per strain, there were strain-dependent variation in inoculation levels and differences in die-off following inoculation (as observed for other strains, and discussed under the single laboratory validation section).

When data for all centrifugation RCFs were combined, colony counts from the supernatant components were lower following 30 min centrifugation than 15 min, and these differences were significant by ANOVA analysis (*C. jejuni* $p < 0.001$, *C. coli* $p = 0.03$; 1 degree of freedom). For *C. jejuni* NZRM 2397, most pairwise comparisons between 30 min and 15 min centrifugation times were significant (Supplementary Table S4). However, differences between RCFs were not significant (*C. jejuni* $p = 0.992$, *C. coli* $p = 0.157$; 2 degrees of freedom) for supernatant components.

Based on the results, subsequent testing using the RCF (modified) method was conducted with an RCF of 4,430 xg centrifugation for 30 min. Although differences between centrifugation RCFs were not significant, 4,430 x g was selected because it gave the highest mean colony counts for *C. jejuni* (for which there was strongest data). The centrifugation parameters found to be optimal were the same as those that were used previously in a longitudinal study that assessed *Campylobacter* numbers on broiler chicken carcasses during primary and secondary processing (Kingsbury et al., 2023). If the RCF modification of the NMD method is implemented for regulatory testing, these results define the range of parameters that should be used in a commercial setting. An RCF setting of 4,430 x g is achievable by most benchtop centrifuges including those used by New Zealand commercial laboratories that conduct testing of poultry rinsates via the NMD programme. In the event that some laboratories do not have this capacity results support that RCF settings of 4,000 and 3,500 x g would be acceptable, but centrifugation times less than 30 min are not recommended.

3.2. Single laboratory comparison of the NMD (reference) and RCF (modified) methods for enumerating and detecting Campylobacter from chicken carcass rinsates

The centrifugation parameters defined in method optimization experiments (RCF of 4,430 x g for 30 min) were used to compare the performance parameters of the RCF (modified) method against the current NMD (reference) method. To inform the method performance for testing poultry rinsates in real-world settings, the method performance compared both the detection and enumeration of poultry isolates in addition to reference strains commonly used in validation studies. Strains were artificially inoculated into chicken carcass rinsates at three inoculation levels and a non-inoculated control. Mean colony counts per sample obtained using the RCF (modified) and NMD (reference) methods, and enrichment results (detection/non-detection), are shown in Table 2.

For each *Campylobacter* strain and each inoculation level, higher counts per plated 2 ml were obtained using the RCF (modified) method relative to the NMD method. Fig. 3 provides a comparison for CFU/2 ml obtained by each method for the high level inoculation samples for each strain, and the relative CFU obtained by the two methods. There was a mean of 6.7-fold (std dev 2.3) higher CFU using the RCF (modified) method. Although colony counts were also higher for the RCF (modified) method for the other inoculation levels, the very low CFU, or fractional recovery of *Campylobacter*, especially when using the NMD (reference) method, meant that relative levels between the two test methods were not calculated.

Campylobacter was also detected from significantly ($p < 0.001$) more samples tested using the RCF (modified) method than the NMD (reference) method for each strain (Tables 2 and 3). From the 126 samples tested, *Campylobacter* was detected in 93 (73.8 %) samples using the RCF (modified) method compared with 65 (51.6 %) using the NMD (reference) method. No samples which were positive using the NMD (reference) method were negative using the RCF (modified) method (negative deviation); conversely, 28 samples were negative by the NMD (reference) method but positive by the RCF (modified) method (positive deviation; Table 3). The mean sensitivity of the RCF (modified) method (SE_{mod}) was 100 compared with 68.8 (std. dev. 15.9) for the NMD (reference) method (SE_{ref}). Furthermore, the limits of detection (LOD_{50} and LOD_{95}) were lower using the RCF (modified) method than the NMD (reference) method (Table 3). The LOD_{50} and LOD_{95}values were in part influenced by the small number of technical replicates tested per strain (which also resulted in large confidence intervals) and the observed inoculum levels. On average, the relative limit of detection (RLOD) was 0.19 (std. dev. 0.16).

For one strain, *C. lari* ATCC 35221, the RCF (modified) method performed less well than for the other strains (although the RCF method still resulted in more CFU obtained and more samples positive than by using the NMD (reference) method, and lower LOD_{50} and LOD_{90} values). Importantly, this is a reference strain rather than a poultry isolate, and *C. lari* in general is isolated infrequently from chicken carcasses during processing. For example, in a New Zealand study of *Campylobacter* isolates obtained from chicken carcasses during primary and secondary processing, there were 1510 isolates of *C. jejuni* (82.5 %), 319 isolates of *C. coli* (17.4 %) and only two of 1831*C. lari* (0.1 %) (Kingsbury et al., 2023). *C. lari* is also isolated far less frequently than *C. jejuni* and *C. coli* from clinical cases of campylobacteriosis (Fitzgerald, 2015; Patrick et al., 2018).

Considering the performance of a method on a range of strains is important because recovery bias and strain variability is common with

Table 2
Mean *Campylobacter* colony counts and detections from inoculated chicken carcass rinsates tested using the RCF (modified), NMD (reference) and enrichment methods.

Strain	Inoculation level (CFU/ml)[1]		NMD		RCF		Enrichment
	Target	Observed	CFU/2 ml (StdDev)	Detection	CFU/2ml[2] (StdDev)	Detection	Detection
C. jejuni							
ATCC33560	0	0	0	0/3	0	0/3	0/3
	0.08	0.06	0	0/3	0.33 (0.58)	1/3	0/3
	0.75	0.59	2.67 (1.53)	3/3	11.67 (3.21)	3/3	–
	45.00	35.60	85.00 (7.00)	3/3	691.33 (27.06)	3/3	–
P1566e	0	0	0	0/3	0	0/3	0/3
	0.08	0.05	0	0/3	0.33 (0.58)	1/3	1/3
	0.75	0.51	0	0/3	2.67 (2.31)	2/3	–
	45.00	30.47	4.33 (1.53)	3/3	24.33 (2.52)	3/3	–
P1539a	0	0	0	0/3	0	0/3	0/3
	0.08	0.14	1.33 (2.31)	1/3	4.67 (2.52)	3/3	3/3
	0.75	1.40	3.00 (1.00)	3/3	18.67 (4.62)	3/3	–
	45.00	84.29	134.33 (14.98)	3/3	936.67 (58.59)	3/3	–
CMB090876	0	0	0	0/3	0	0/3	0/3
	0.08	0.15	0	0/3	1.00 (1.00)	2/3	1/3
	0.75	1.47	2.67 (2.08)	3/3	12.00 (2.65)	3/3	–
	45.00	88.41	124.00 (22.87)	3/3	714.33 (9.02)	3/3	–
CMB09552	0	0	0	0/3	0	0/3	0/3
	0.08	0.13	1.33 (1.53)	2/3	1.33 (0.58)	3/3	2/3
	0.75	1.28	3.33 (1.15)	3/3	19.67 (2.31)	3/3	–
	45.00	76.63	127.67 (20.21)	3/3	876.67 (49.33)	3/3	–
PL0183	0	0	0	0/3	0	0/3	0/3
	0.075	0.18	0.33 (0.58)	1/3	2.00 (1.00)	3/3	2/3
	0.75	1.82	4.67 (1.53)	3/3	39.33 (7.37)	3/3	–
	45.00	109.21	222.00 (30.32)	3/3	2006.67 (23.09)	3/3	–
C. coli							
ATCC33559	0	0	0	0/3	0	0/3	0/3
	0.08	0.02	0	0/3	1.00 (1.00)	2/3	3/3
	0.75	0.16	0.33 (0.58)	1/3	4.00 (2.65)	3/3	–
	45.00	4.81	8.67 (1.53)	3/3	91.00 (10.58)	3/3	–
P1572b	0	0	0	0/3	0	0/3	0/3
	0.08	0.13	0.33 (0.58)	1/3	2.00 (2.65)	2/3	2/3
	0.75	1.31	3.33 (2.08)	3/3	28.33 (6.11)	3/3	–
	45.00	78.67	152.00 (16.46)	3/3	1333.33 (213.62)	33	–
CMB09566	0	0	0	0/3	0	0/3	0/3
	0.08	0.06	0	0/3	1.00 (0.00)	3/3	1/3
	0.75	0.60	2.33 (1.15)	3/3	19.33 (5.51)	3/3	–
	45.00	36.01	83.00 (7.55)	3/3	532.33 (65.58)	3/3	–
C. lari							
ATCC35221	0	0	0	0/3	0	0/3	0/3
	0.08	0.10	0	0/3	0.33 (0.58)	1/3	3/3
	0.75	0.95	0.67 (1.15)	1/3	2.00 (1.73)	3/3	–
	45.00	57.27	68.00 (3.61)	3/3	70.33 (39.88)	3/3	–
PL0385	0	0	0	0/3	0	0/3	0/3
	0.08	0.05	0	0/3	0	0/3	3/3
	0.75	0.47	0.33 (0.58)	1/3	5.67 (1.53)	3/3	–
	45.00	28.43	31.33 (1.53)	3/3	221.00 (22.54)	3/3	–
CMB91863	0	0	0	0/3	0	0/3	0/3
	0.08	0.04	0	0/3	0.33 (0.58)	1/3	1/3
	0.75	0.40	0	0/3	3.33 (0.58)	3/3	–
	45.00	23.74	43.67 (2.08)	3/3	217.33 (11.93)	3/3	–
Total detections[3]				65/126 (51.6 %)		93/126 (73.8 %)	22/54 (40.7 %)

[1] The rinsate volume inoculated was 400 ml, but the inoculum level per ml was presented for easier comparability of results across methods. The observed inoculation level was determined by plating dilutions of the inoculum at the time of rinsate inoculation.
[2] Where colony counts were > 150 per plate from plating of 6 × 0.33 ml, counts from plating of 2 × 100 μl were used and multiplied by 10.
[3] For the 0 CFU/ml inoculation level control, one set of controls was tested for each set of two strains tested per day; hence the denominator is not the sum of all entries in the column.

all methods used to detect and enumerate *Campylobacter*, including enumeration on mCCDA agar relative to other plating media (Dziegiel et al., 2024; Hazeleger et al., 2022; Hetman et al., 2020; Kramer et al., 2000; Ladely et al., 2017; Oyarzabal et al., 2005). *Campylobacter* strain-dependent variations are also widely documented due to differences in stress tolerance, such as to cold and oxygen stress (Habib et al., 2010; Kaakoush et al., 2007; Oh et al., 2019). Differences in stress tolerance likely contributed to the strain-dependent differences in inoculation levels and how well strains were recovered relative to the inoculation levels (also observed in method optimization experiments), independent of the enumeration method.

Culture enrichment is a recommended procedure for the detection of *Campylobacter* where present in low numbers, and/or where *Campylobacter* cells may be stressed (International Organization for Standardization, 2017). Therefore, enrichments were also performed on uninoculated and low-level (target 30 CFU/400 ml) samples (Table 2). *Campylobacter* was not recovered from uninoculated controls using any method. Importantly, the RCF (modified) and enrichment methods gave equivalent detection results. Of the 36 low-level inoculated samples, *Campylobacter* was detected from the same number of samples using both methods (22 samples each; 61.1 %), although the same samples were not always positive by both methods. For example, more low-level *C. jejuni*-inoculated samples tested positive by the RCF (modified) method (13/18) compared with 9/18 positive by enrichment, while less

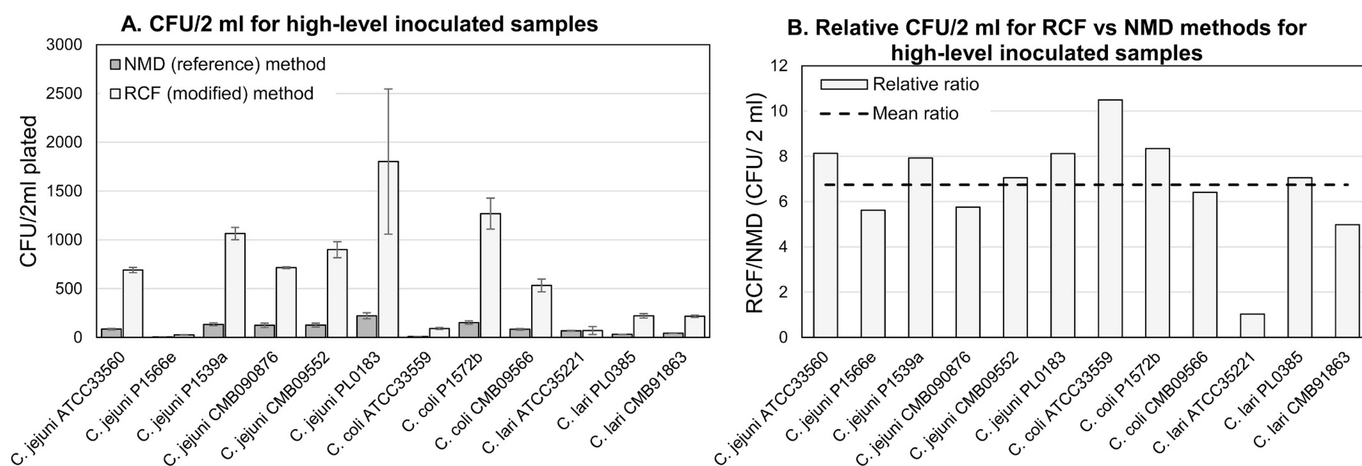

Fig. 3. Comparison of the CFU/2 ml (A) and relative counts (B) for the NMD (reference) and RCF (modified) methods for different *Campylobacter* strains. Shown are data for the high level inoculated samples.

low-level *C. lari*-inoculated samples were positive by the RCF method (2/9) compared with 7/9 using enrichment. In contrast, only 5 low-level inoculated samples (13.9 %) tested positive using the NMD (reference) method; the differences between methods were statistically significant ($p < 0.001$). The improved detection using enrichment over direct plating (the NMD reference method) was expected and likely due to stressed cells being able to recover during enrichment, but not able to grow when plated onto mCCDA directly. In addition, results would be influenced by the differing volume of rinsate tested by each method; a five-fold higher volume of rinsate was added to enrichments (10 ml) relative to direct plating via the NMD (reference) method (2 ml). For the RCF (modified) method, if stressed cells were present, there would have been more stressed and non-stressed cells plated overall compared with the NMD and enrichment methods due to the centrifugation concentration.

Taken together, results showed that the RCF (modified) method was more sensitive and could enumerate chicken carcass rinsates with lower levels of *Campylobacter* than the detection limit of the current NMD (reference) method, when conducted under a single laboratory validation. Specifically, there were 6.7-fold higher CFU per plate for high-level inoculated samples using the RCF (modified) method, *Campylobacter* was detected from significantly more samples inoculated at different concentrations to examine fractional recovery, and the mean LOD_{50} and LOD_{90} were 19 % (RLOD 0.19) of those tested using the NMD (reference) method. Importantly, the RCF (modified) method was also effective for all New Zealand poultry *Campylobacter* isolates tested, which covered a wide range of genotypic and presumably, phenotypic, variability.

3.3. Assessment of cells lost in discarded supernatant

The detection limit of the RCF (modified) method if all CFU were retained in the cell pellet fraction is 20 CFU per 400 ml chicken carcass rinsate, which is ten-fold lower than the NMD (reference) method. However, as observed in these experiments, some *Campylobacter* CFU were present in the discarded supernatant. Understanding the proportion of CFU retained in the pellet is important for providing a better estimate of the RCF (modified) method detection limit, and consequently more accurate correction factor for calculation of CFU present in the entire 400 ml chicken carcass rinsates. The percentage recovery was calculated below using two approaches; comparing the CFU present in the 5 ml pellet fraction relative to the 5 ml pellet and 45 ml supernatant fractions, and relative to the CFU present in 50 ml as calculated from direct plating. A further approach would be to compare CFU relative to inoculation levels; however, for some strains, the CFU obtained using both methods was much lower than input levels, which we attribute to *Campylobacter* becoming stressed by components present in the chicken carcass rinsate prior to plating (as discussed earlier). This approach was not taken because it does not provide a direct comparison between the NMD and RCF methods, which was the purpose of the study.

The percentage recovery was first calculated by comparing the estimated CFU from the pelleted component (calculated for 5 ml) relative to the estimated CFU present in the 50 ml based on CFU from the supernatant and pelleted components. Two ml volumes of supernatant from high-level inoculum samples (target 18,000 CFU/400 ml rinsate) were plated to provide an assessment of the CFU lost in the discarded supernatant. Mean percentage recoveries for individual strains ranged from 65.6 to 88.7 % depending on the strain tested (Fig. 4, dark grey bars; Supplementary Table S5, final column). Where inoculum levels were lower than intended (for example, for *C. lari* ATCC 35221 and *C. jejuni* P1566e) these calculations were based on very low counts from the supernatant fraction. The mean percentage recovery for all strains was 74.3 % (std. dev. 7.7 %), which equates to 25.7 % loss of cells in the discarded supernatant. Based on this data, the *Campylobacter* CFU present in samples were on average 1.3 times higher than those obtained by plating the pelleted fraction, which gives a RCF method detection limit of 26. The percentage recovery for the pelleted component was also calculated relative to counts obtained from NMD (reference) method direct plating. Percentage recoveries ranged from 10.3 to 105.0 % (Fig. 4, light grey bars; Supplementary Table S5, penultimate column). For individual strains, the calculation is of limited value where there are very low CFU from direct plating. However, where data were more robust, and where percentage recovery using this calculation is lower than that based on pellet and supernatant CFU (final column, Supplementary Table S5), this may indicate reduced recovery due to additional factors such as die off during centrifugation, or cell clumping. For example, *C. lari* ATCC 35221 percentage recovery was lower relative to direct plating estimates than to supernatant plus pellet estimates. In contrast, percentage recovery for *C. jejuni* ATCC 33560 was similar by both methods, suggesting that the majority of CFU loss during the RCF method was due to cells being discarded in the supernatant for this strain.

When results for all strains were considered together, the mean percentage recovery relative to CFU/50 ml calculated from direct plating (the NMD method) was 70.3 % (std. dev. 23.3 %). This equates to 29.7 % of cells either retained in the discarded supernatant, dying off or becoming stressed during centrifugation, or underestimated due to cell clumping. Based on the percentage recovery relative to direct plating, the *Campylobacter* CFU present in samples were on average 1.4 times higher than those obtained by plating the pelleted fraction, which gives a RCF method detection limit of 28 (conversion factor 1.4 multiplied by

Table 3
Performance parameters of the NMD (reference) method compared with the RCF (modified) method.

	C. jejuni						*C. coli*			*C. lari*			
	ATCC 33,560	P1566e	P1539a	CMB 090876	CMB 09552	PL0183	ATCC 33,559	P1572b	CMB09566	ATCC 35,221	PL0385	CMB 91,863	*Total*
Positive agreement (PA)[1]	6	3	7	6	8	7	4	7	6	4	4	3	*65*
Negative agreement (NA)[1]	5	6	3	4	3	3	4	4	3	5	6	5	*51*
Positive deviation (PD)[1]	1	3	2	2	1	2	4	1	3	3	2	4	*28*
Negative deviation (ND)[1]	0	0	0	0	0	0	0	0	0	0	0	0	*0*
													Mean (StdDev)
Sensitivity of NMD (reference) method (SE_{ref}): (PA + ND)/(PA + ND + PD) x 100	85.7	50.0	77.8	75.0	88.9	77.8	50.0	87.5	66.7	57.1	66.7	42.9	*68.8 (15.9)*
Sensitivity of RCF (modified) method (SE_{mod}): (PA + PD)/(PA + ND + PD) x 100	100	100	100	100	100	100	100	100	100	100	100	100	*100 (0.0)*
LOD_{50} NMD (confidence interval) (CFU/ml)	0.18 (0.05–0.64)	5.27 (0.79–35.26)	0.22 (0.05–0.96)	0.43 (0.12–1.57)	0.09 (0.02–0.37)	0.28 (0.06–1.25)	0.33 (0.05–2.35)	0.20 (0.05–0.90)	0.18 (0.05–0.64)	1.84 (0.25–13.51)	0.92 (0.13–6.74)	4.11 (0.62–27.45)	*1.17 (1.73)*
LOD_{50} RCF (confidence interval) (CFU/ml)	0.09 (0.02–0.41)	0.24 (0.07–0.87)	0.03 (0.01–0.15)	0.10 (0.02–0.42)	0.03 (0.01–0.13)	0.03 (0.01–0.20)	0.02 (0.00–0.06)	0.09 (0.02–0.38)	0.02 (0.00–0.06)	0.15 (0.03–0.65)	0.18 (0.05–0.64)	0.28 (0.06–1.25)	*0.10 (0.09)*
LOD_{95} NMD (confidence interval) (CFU/ml)	0.76 (0.21–2.75)	22.79 (3.40–152.40)	0.93 (0.21–4.16)	1.86 (0.51–6.79)	0.37 (0.09–1.58)	1.20 (0.27–5.40)	1.40 (0.19–10.16)	0.87 (0.20–3.89)	0.77 (0.21–2.78)	7.95 (1.08–58.39)	3.97 (0.54–29.12)	17.75 (2.66–118.63)	*5.05 (7.49)*
LOD_{95} RCF (confidence interval) (CFU/ml)	0.41 (0.09–1.76)	1.03 (0.28–6.87)	0.12 (0.02–0.64)	0.42 (0.10–1.82)	0.11 (0.02–0.57)	0.14 (0.02–0.87)	0.06 (0.02–0.25)	0.38 (0.09)-1.62)	0.07 (0.02–0.26)	0.64 (0.15–2.83)	0.76 (0.21–2.75)	1.20 (0.27–5.40)	*0.45 (0.39)*
Relative limit of detection (RLOD): (LOD_{50} RCF)/(LOD_{50} NMD)	0.53	0.05	0.13	0.23	0.29	0.12	0.05	0.43	0.09	0.08	0.19	0.07	*0.19 (0.16)*

[1] Positive agreement: samples positive by both methods; Negative agreement: samples negative by both methods; Positive deviation: samples negative by NMD but positive by RCF; Negative deviation: samples positive by NMD but negative by RCF.

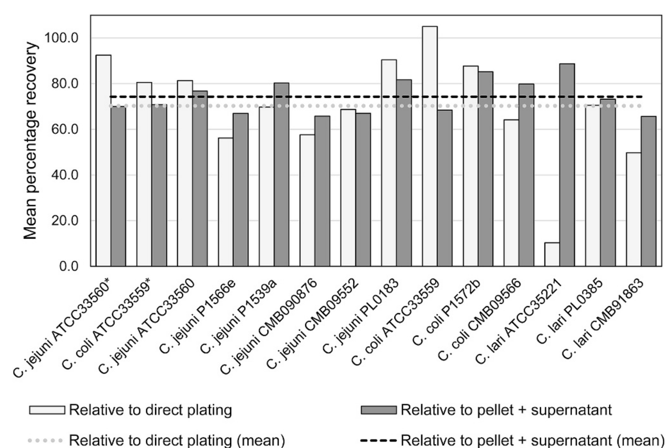

Fig. 4. Mean percentage recovery of colony forming units (CFU) retained in the 5 ml pelleted fraction relative to the CFU present in 50 ml determined by direct plating, or present in the pellet and supernatant fractions combined. Data were from samples with a target inoculum of 18,000 CFU/400 ml. Data for the strains marked with an asterisk were based on five replicates (method optimization experiments); all other data were based on three replicates (single laboratory validation experiments).

dilution factor 20). This provides a more conservative estimate than that based on the pellet and supernatant fractions because it takes into account the low recovery of the *C. lari* ATCC 35221 strain relative to the NMD method. This RCF method detection limit of 28 was applied in subsequent experiments for calculating colony counts per 400 ml rinsate based on the 2 ml plated from the RCF pelleted fraction. A similar percentage loss was observed in earlier experiments using *C. jejuni* ATCC 33560 (data not provided), based on which a RCF method detection limit of 29 was previously applied for calculating CFU/400 ml chicken carcass rinsate (Kingsbury et al., 2023).

Currently, the NMD programme reports findings as CFU per carcass (which technically refers to the carcass rinsate because not all *Campylobacter* cells will be removed from carcasses during the rinse procedure). If *Campylobacter* is not detected, a value of 100 CFU per carcass rinsate is reported for statistical purposes, which is half the detection limit of the NMD method. If the RCF modification to the method is implemented, a method detection limit of 28 CFU/400 ml rinsate would be applied, and a value of 14 CFU per carcass rinsate (50 % of the RCF method detection limit) would be reported if *Campylobacter* was not detected. A point to note is that the detection limit of 200 for the NMD method means that small differences in CFU numbers would result in larger differences in calculated CFU per rinsate relative to those calculated using the RCF method. As such, the RCF method would have a higher level of precision than the NMD method, which is a measure of the closeness of agreement between independent test results under stipulated conditions. Applying the observed RCF method detection limit would also be useful for estimating CFU per carcass rinsate for purposes such as data modelling.

The observed CFU loss following supernatant removal, even under controlled and careful experimental conditions, highlights the importance of careful handling of centrifuged tubes, removing the supernatant without disturbing the pellet immediately following centrifugation, for optimal performance of the method. Once the supernatant has been discarded, effective resuspension of the pellet in the retained supernatant before plating is also necessary to ensure that no cell aggregation occurs.

3.4. Aerobic plate counts of chicken carcass rinsates

Background microflora present in rinsates might compete with *Campylobacter* present and therefore influence recovery. For example, centrifugation would also concentrate cefoperazone-resistant bacteria such as extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* that can cause overgrowth on mCCDA plates (Chon et al., 2016). However, the occurrence of ESBL-producing Enterobacteriaceae is currently low in New Zealand, and they were not detected in a recent survey of *E. coli* and enterococci from food animals (Cornelius et al., 2024). The levels of background flora were tested for each rinsate batch used for single laboratory validation testing on the same day that validation testing was conducted. For all batches, the APC counts were very low (<10 CFU/ml). The low numbers reflect the efficacy of antimicrobial steps during primary processing and may also be influenced by freezing of the rinsates. The very low number of background microflora was considered unlikely to have influenced *Campylobacter* counts in this study. In addition, no overgrowth of non-*Campylobacter* bacteria were observed, nor detected following colony confirmation.

3.5. Method comparison under commercial conditions

A total of 863 naturally contaminated chicken carcass rinsate samples arising from regulatory testing of commercial flocks were tested with the NMD (reference) method alongside the RCF (modified) method under commercial conditions at two laboratories. Significantly more samples ($p < 0.001$) tested positive using the RCF (modified) method (257/863; 29.8 %) compared with 114 (13.2 %) positive samples using the NMD (reference) method (Table 4). There were 155 samples which were positive by the RCF (modified) method but negative by the NMD (reference) method. Of these, 88.4 % had 1–7 CFU over the six plates (2 ml) which is below the detection limit of the NMD (reference) method (<200 CFU/rinsate) when the CFU/400 ml rinsate was calculated based on a correction factor of 28 (Fig. 5A, Supplementary Table S6). A majority of the samples that were positive by the NMD (reference) method (102/114; 89.5 %) were also positive by the RCF (modified) method. Of the 12 samples that were only positive by the NMD (reference) method, 11 had a single colony and one sample had two (therefore, at or near the LOD for the NMD reference method). The sensitivity of the RCF (modified) method was higher (95.5 %) than the NMD (reference) method (42.4 %) (Table 4). Therefore, the RCF (modified) method outperformed the NMD (reference) method for commercial samples where *Campylobacter* was present in low levels.

Current regulatory limits for premises require no more than two carcasses with >6,000 CFU/carcass rinsate per sampling window (Ministry for Primary Industries, 2023). Detection limits of 200 for the NMD (reference) method and 28 for the RCF (modified) method (as defined above) were employed to calculate the CFU per 400 ml chicken carcass rinsate; the same six samples tested using both methods returned counts of >6,000 CFU/carcass rinsate (Fig. 5B, Supplementary Table S6). Therefore, both methods gave equivalent results when enumerating high-level positive samples.

4. Conclusions

The RCF (modified) method detected genotypically diverse strains of

Table 4
Method comparison between NMD (reference) and RCF(modified) methods for detection of *Campylobacter* from commercial chicken carcass rinsates.

Parameter	Total (%)
Samples tested	863 (100.0)
Positive by NMD method	114 (13.2)
Positive by RCF method	257 (29.8)
Positive agreement	102 (11.8)
Negative agreement	594 (68.8)
Positive deviation	155 (18.0)
Negative deviation	12 (1.4)
Sensitivity of NMD method: (PA + ND)/(PA + ND + PD) x 100	42.4
Sensitivity of RCF method: (PA + PD)/(PA + ND + PD) x 100	95.5

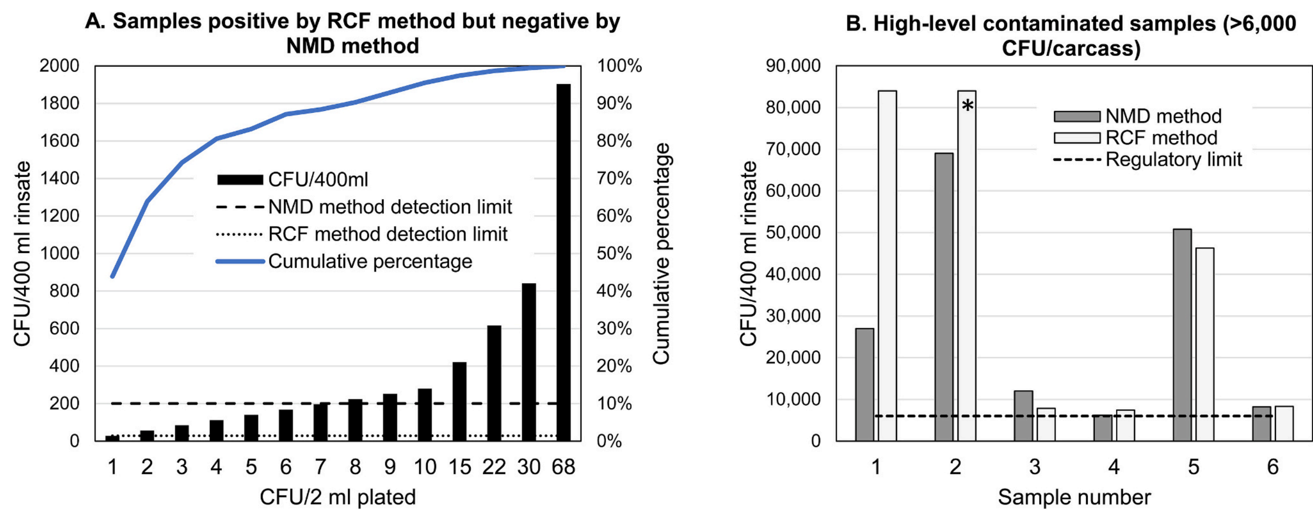

Fig. 5. Method comparison of NMD (reference) and RCF (modified) methods under commercial conditions. A. Colony forming units (CFU) for 155 commercial chicken carcass rinsate samples for which *Campylobacter* was detected using the RCF (modified) method but not detected using the NMD (reference) method. Shown are the CFU recorded per 2 ml plated, the calculated CFU/400 ml rinsate, and the cumulative percentage of samples with the respective CFU or less. B. The calculated CFU/400 ml rinsate for the six samples which were higher than the regulatory limit (6000 CFU/400 ml rinsate) when tested by both methods. For sample 2 tested by the RCF method, the CFU were too numerous to count for the highest dilution plated; thus the CFU/400 ml rinsate were higher than that depicted (as indicated by the asterisk).

Campylobacter from artificially contaminated chicken carcass rinsate samples with a higher sensitivity and greater prevalence relative to the NMD (reference) method. As well as being more effective than the current NMD method, any modification should also be practicable and cost-effective for testing laboratories. The centrifugation parameters applied are attainable using conventional table-top centrifuges common-place in laboratories that test commercial poultry rinsate samples in New Zealand, and the RCF method was also demonstrated to outperform the NMD method for testing naturally contaminated chicken in this setting. The results support the implementation of the RCF method as a modification of the reference NMD method to estimate the lower levels of *Campylobacter* contamination in rinsates which are not detected by the current NMD method. Improved *Campylobacter* detection from poultry carcasses is expected to provide benefit for regulatory purposes, and support the *Campylobacter* Action Plan to minimize foodborne campylobacteriosis attributable to poultry meat in New Zealand.

CRediT authorship contribution statement

Joanne M. Kingsbury: Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Anne Midwinter:** Writing – review & editing, Methodology, Conceptualization. **John Mills:** Writing – review & editing, Methodology, Conceptualization. **Mark Englefield:** Writing – review & editing, Methodology, Conceptualization. **Roy Biggs:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Anne-Marie Perchec Merien:** Writing – review & editing, Project administration, Methodology, Funding acquisition, Conceptualization. **Nicola Dermer:** Writing – review & editing, Project administration, Methodology, Funding acquisition. **Aswathi Soni:** Writing – review & editing, Project administration. **Milana Blakemore:** Writing – review & editing, Project administration, Methodology, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:
Joanne Kingsbury, Anne Midwinter, John Mills, Mark Englefield, Aswathi Soni, Nicola Dermer, Anne-Marie Perchec Merien, Milana Blakemore reports administrative support was provided by New Zealand Food Safety Science and Research Centre. Joanne Kingsbury reports equipment, drugs, or supplies was provided by Van den Brink Poultry Ltd. Joanne Kingsbury, Anne Midwinter, John Mills, Mark Englefield reports financial support was provided by New Zealand Ministry for Primary Industries. 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.

Acknowledgements

The authors gratefully acknowledge the Tegel laboratories and technical team members for conducting testing of commercial chicken carcass rinsate samples for method comparisons (Maree Callander, Sheryl Bellringer, Alison Cook, Paula O'Connell-Taylor, James Pope, Joanna McLellan, Fiona Chen, Michael Fenton, Rowan Tunnicliff-Roberts, Heather Rayner, Hayley Erskine, Fiona Christial, Sunny Huang, Kay Wang and Kokwai Fung). We thank Van Den Brink Poultry Ltd., Christchurch Compliance and Quality Assurance Manager Nicola McKeown and staff (Michelle Radcliffe, Cristie Hill, Cheryl Buttolph, Robyn Moses and Sharon Cane) for collecting and supplying chicken carcass rinsates. We also thank the Poultry Industry Association of New Zealand for supplying consumables for rinsate acquisition. We appreciate the laboratory support from New Zealand Institute for Public Health and Forensic Science (PHF Science) Public Health laboratory technicians Kirstin Thom, Ashley Orton, Alice Chechi and Tori Head, as well as statistical analyses conducted by Bridget Armstrong (PHF Science). We also thank Iain Haysom (PHF Science), Daniel Bohnen (PHF Science) and Phil Bremer (University of Otago, New Zealand Food Safety Science and Research Centre) for critical evaluation of the manuscript.
The study was funded by the Food Safety Operational Research Programme at the New Zealand Ministry for Primary Industries through the New Zealand Food Safety Science and Research Centre.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2025.107207.

Data availability

Data will be made available on request.

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