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Digital Droplet-PCR for Quantification of Viable *Campylobacter jejuni* and *Campylobacter coli* in Chicken Meat Rinses

Janani Govindaswamy [1,†], Véronique Zeller-Péronnet [1,*,†], Melanie Pavlovic [1], Daniela Wirtz [1], Larissa Murr [1], Diana Thärigen [1], Bertrand Colson [2], Steffen Uhlig [2], Ulrich Busch [1] and Ingrid Huber [1]

[1] Bavarian Health and Food Safety Authority (LGL), 85764 Oberschleissheim, Germany; janani.govindaswamy@mri.bund.de (J.G.); melanie.pavlovic@lgl.bayern.de (M.P.); daniela.wirtz@lgl.bayern.de (D.W.); larissa.murr@lgl.bayern.de (L.M.); diana.thaerigen@lgl.bayern.de (D.T.); ulrich.busch@lgl.bayern.de (U.B.); ingrid.huber@lgl.bayern.de (I.H.)

[2] QuoData GmbH, Quality & Statistics, 01309 Dresden, Germany; colson@quodata.de (B.C.); steffen.uhlig@quodata.de (S.U.)

* Correspondence: veronique.zeller-peronnet@lgl.bayern.de; Tel.: +49-9131-6808-5505

† These authors contributed equally to this work.

Featured Application: Droplet digital PCR (ddPCR) in combination with propidium monoazide (PMA) treatment provides a method that is accurate, efficient, and reliable and that can be easily and quickly applied in routine use to quantify *Campylobacter jejuni* and *coli* in chicken meat rinses from slaughterhouse and retail.

Abstract: The EU commission established Regulation (2017/1495) in 2017 to reduce *Campylobacter* on chicken skin and to decrease the number of human cases of campylobacteriosis attributable to the consumption of poultry meat. A Process Hygiene Criterion based on colony-forming unit data was set to a maximum of 1000 CFU *Campylobacter* spp. per gram chicken neck skin at slaughterhouses. Confronted with stressors, including cold, oxidative stress or antibiotic treatment, live cells may enter into a viable but non-cultivable state (VBNC) and lose the ability to grow, in reference to the plate count ISO 10272-2:2017 method, but still possess the potential to recover and cause infections under favorable conditions. In this study, a droplet digital PCR combined with the intercalating dye propidium monoazide (PMA) was established for quantification of *C. coli* and *C. jejuni* in chicken meat rinses. The PMA was used to inactivate DNA from dead cells in this technique. This method was successfully validated against the reference method according to ISO 16140-2:2016 for accuracy and relative trueness. Additionally, it presented a 100% selectivity for *Campylobacter jejuni* and *C. coli*. Moreover, the technical measurement uncertainty was determined according to ISO 19036:2019, and the applicability of ddPCR for quantifying *C. coli* and *C. jejuni* in chicken meat rinses was investigated on naturally contaminated samples from slaughterhouses and supermarkets. Results obtained from this study demonstrated a strong correlation to qPCR as well as the classical microbiological reference method.

Keywords: food safety; *Campylobacter jejuni* and *coli*; chicken skin; droplet digital PCR (ddPCR); propidium monoazide (PMA); molecular quantification; routine analysis

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1. Introduction

Human campylobacteriosis is the most reported foodborne illness in the European Union (EU) since 2005, which is commonly attributed to broiler meat contamination [1]. In 2018, campylobacteriosis was the most commonly reported zoonosis, per se representing almost 70% of all the reported cases [2]. Regarding foodborne diseases, campylobacteriosis has shown the highest hospitalization rate in the years between 2015 and 2019, with

a stable count of approximately 20,000 cases per year [3]. *C. jejuni*, followed by *C. coli* are the predominant thermophilic *Campylobacter* species in poultry samples, which are mainly responsible for foodborne human infections [4,5]. A Process Hygiene Criterion (PHC) of a maximum of 1000 Colony Forming Units (CFU) thermophilic *Campylobacter* per gram of chicken skin came into effect in January 2018 (Commission Regulation (EU) 2017/1495 [6]), to reduce significantly the *Campylobacter* contamination of poultry at slaughterhouses. According to the estimation of EFSA [3], more than 50% of the public health risk due to *Campylobacter* could be reduced if none of the carcasses would exceed this PHC. The reference colony-enumeration method [7] for the quantification of *Campylobacter* is time-consuming, laborious, and it can underestimate viable and potentially infectious cells. Environmental conditions such as cold, oxidative stress [8,9], as well as antibiotic treatments [10,11] have been reported to completely stop the growth of *Campylobacter* on agar plates. This may be due to the fact that cells enter into a viable but non-cultivable state (VBNC) [12]. The VBNC state is a survival strategy for bacteria in order to overcome the above mentioned stressful external conditions [13]. Under favorable conditions, VBNC cells can revive and recover full infectious ability [14,15]. Both cultivable and VBNC cells represent a significant threat to public health. While cultivable *Campylobacter* can be quantified by plate counting, VBNC remains undetected, making the reference method unsuitable for a proper risk assessment for *Campylobacter* spp. in broiler meat. Molecular-based technology, like quantitative real-time PCR (qPCR) and digital PCR (dPCR) can overcome this limitation by detecting and quantifying *Campylobacter* DNA of VBNC cells. Furthermore, a sample treatment prior to DNA extraction analysis with DNA-intercalating dyes like propidium monoazide (PMA) enables differentiation between viable and dead cells via PCR. PMA passes through membrane-compromised dead cells, intercalates into cytoplasmic DNA, upon light exposure crosslinks to DNA, and inactivates it for PCR amplification [9,16,17]. Therefore, only intact and putatively infectious units (IPIU) comprising CFU and VBNC *Campylobacter* cells generate a signal in qPCR. A viability PMA-qPCR (v-qPCR) has been recently published [18] and it integrates a dead-cell standard to compensate for the variable residual PCR signal of dead cells [19,20]. As [21] reported in 2019, v-qPCR allows an efficient and a reliable quantification of viable thermophile *Campylobacter*.

Digital PCR (dPCR), as the third generation of PCR technology has been proven to offer a new approach for its absolute quantification of copies of target molecules [22,23]. It offers an alternative to qPCR to obtain precise quantification of nucleic acids without a standard curve, and it is not dependent on the number of amplification cycles to determine the initial amount of nucleic acids in the sample [24,25]. Chamber-based dPCR (cdPCR) and droplet-based dPCR (ddPCR) are presently two basic approaches for conducting dPCR. In ddPCR, the compartmentalization of the reaction mix is achieved by using plastic-consumables (generator cartridges) with ultra-thin capillaries (microfluidics) in order to prepare a water-in-oil emulsion prior to the PCR; heat stable droplets are formed [26]. Microfluidics, i.e., miniaturization of fluid-handling, has enabled the massively-parallel sample partitioning and the advent of dPCR platforms [27]. In ddPCR, the sample is partitioned into approximately 20,000 droplets before the PCR cycles, subsequently, each droplet consists of ideally single (or few) copies of the target molecules and some others contain no copy of target molecules [28]. The absolute number of target nucleic acid molecules contained in the original sample before partitioning can be calculated from the ratio of positive events to total partitions, using binomial Poisson statistics [24]. Partitioning the sample into droplets before PCR holds the potential to overcome the inhibitory effects present in the sample matrix [28]. Finally, ddPCR enables the detection and the quantification of even low copy numbers due to the independent end-point PCR reaction occurring on every droplet from the sample [29].

In the current study, the combination of the sample pre-treatment with PMA dye was explored prior to DNA extraction and quantification of viable *C. jejuni* and *C. coli* in a viability ddPCR (v-ddPCR). The v-ddPCR method was optimized successfully in-house, validated according to ISO 16140-2:2016 [30], and evaluated on naturally contaminated

chicken samples. The results showed a significant correlation between the v-ddPCR, the reference microbiological ISO [7], and the recently published v-qPCR methods [18]. Furthermore, the technical measurement uncertainty was assessed based on ISO 19036:2019 [31], using QuoData statistical analysis. Finally, v-ddPCR may be used as an additional technique for rapid and easy monitoring in routine applications.

2. Materials and Methods

2.1. Strains and Growth Conditions

C. jejuni NCTC 11168 (National Collection of Type Cultures, Salisbury, UK) and *C. coli* ATCC 43478 (American Type Culture Collection) were used as reference strains for method validation concerning $LOD_{95\%}$, precision, and LOQ.

For the inclusivity study, *C. jejuni* and *C. coli* isolates were collected from Bavarian slaughterhouses and supermarket samples, which were considered for routine analysis at the LGL (Bavarian Health and Food Safety Authority). In the scope of cooperation projects, additional isolates were provided to the LGL by BfR (German Federal Institute for Risk Assessment). The identity of all isolates was verified at the species level prior to DNA extraction using a MALDI Biotyper (Bruker Daltonics, Bremen, Germany) according to the study from Huber et al. [32]. Moreover, all isolates were sequenced using a next-generation sequencing platform at LGL (data not shown). All *Campylobacter* isolates or strains were cultivated on Tryptone Soy Agar with Sheep Blood (Thermo Fisher Scientific Inc., Waltham, MA, USA) and then incubated for 44 h ± 4 h at 42 °C under microaerobic conditions. The cultivation of non-*C. jejuni* and non-*C. coli* strains were carried out according to the growth conditions specified by the culture collections DSMZ (German Collection of Microorganisms and Cell Cultures) and ATCC (American Type Culture Collection) or according to BfR recommendations.

The IDs of the individual isolates and strains used for inclusivity and exclusivity determinations are listed in Supplementary Materials List S1.

2.2. Live and Dead Cell Standards

Live and dead cell standards consist of live or dead *Campylobacter* cells (*C. coli* or *C. jejuni*) in a specific concentration, and they were used to spike the samples. These cell standards were essential for the in-house validation (accuracy, trueness, and measurement uncertainty). Three standards that were provided by BfR were: *C. jejuni* live-cell standard (NCTC 11168, 4.6×10^6 CFU/mL), *C. jejuni* dead-cell standard (DSM 4688, 1×10^9 bacterial counts/mL), and *C. coli* live-cell standard (WDCM 00004, 7×10^5 CFU/mL). The *C. coli* dead cell standard (ATCC 43478, 1×10^9 bacterial counts/mL) was prepared at LGL according to the BfR protocol [33]. Live- and dead-cell standards were stored at −80 °C, thawed at room temperature for 15 min, and kept on ice for 30 min prior to the artificial spiking.

2.3. Raw Meat Samples and Rinses

For each sample, 10 g of raw meat were collected randomly and weighed in a BagPage + filter bag with a pore size of 280 µm (Interscience Lab Inc., Woburn, MA, USA). The 10 g samples were diluted (1:10) in 1% buffered peptone water (Merck KGaA, Darmstadt, Germany). An additional 10 g sample was weighed and diluted (1:2) in buffered peptone water. After homogenization in a stomacher for 2 min, the meat rinse was carefully transferred to a 50 mL falcon tube. Two kinds of rinses were prepared:

(A) *Campylobacter*-free rinses were spiked with live and dead cell standards, and they were used for method comparison study (accuracy and relative trueness), as well as technical measurement uncertainty study. The absence of live and dead *Campylobacter* cells was confirmed previously in-house based on the three methods described in Sections 2.7, 2.8 and 2.10. Each of the spiked meat rinses was divided into three aliquots, 1 mL for ddPCR with PMA, 1 mL for ddPCR without PMA, and 1 mL for the microbial reference method. The v-ddPCR results were compared to the results of the reference method [7];

(B) Rinses prepared with naturally contaminated routine samples from LGL (2020) and retail samples (2019 to 2021) were used to investigate the applicability of v-ddPCR for quantifying *Campylobacter* in chicken rinses. The LGL routine samples consist of 19 chicken neck skin (NS) from Bavarian slaughterhouses and 13 chicken breast meat (BM, within the scope of zoonosis monitoring) from Bavarian retail. Additionally, 25 chicken meat retail samples (RS) like chicken neck skin, chicken breast meat, chicken thighs, chicken wings, and chicken drumstick were bought in supermarkets between October 2019 and March 2021 in the region of Munich. Five 1 mL aliquots were needed for each rinse: 1 mL was used for the quantification of *Campylobacter* with the microbiological reference assay, two times 1 mL for qPCR (with and without PMA treatment), and two times 1 mL for ddPCR (with and without PMA treatment). The v-ddPCR results were compared to the results of the reference method [7] and the v-qPCR [18].

All meat rinse IDs are listed in Supplementary Materials List S2.

2.4. DNA Extraction

DNA from *Campylobacter* isolates and meat rinses was extracted using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's protocol. Finally, DNA was eluted in 75 µL elution buffer for ddPCR and 100 µL elution buffer for the qPCR assay, respectively. Alternatively, a PureLink Genomic DNA Mini Kit (Thermo Fisher Scientific Inc.) or a High Pure PCR Template Kit (Merck KGaA, Darmstadt, Germany) were applied according to the manufacturer's instructions for the extraction of DNA from non-*Campylobacter* strains in the context of exclusivity determination.

2.5. DNA Quantification

DNA from pure bacterial cultures that was used for the exclusivity and the inclusivity studies was quantified using a Qubit Fluorometer and the Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific Inc.) according to the manufacturer's instructions. The DNA concentration was adjusted to the concentration of 10 pg/µL for ddPCR analysis by using sonicated salmon sperm DNA (10 ng/µL) (Thermo Fisher Scientific Inc.).

2.6. Oligonucleotides for ddPCR and qPCR

The duplex v-ddPCR method, including two detection systems in the FAM channel based on specific single-copy genes for *C. jejuni* and *C. coli*, was combined with an internal amplification control (IAC) in the HEX channel. The species-specific target genes were selected based on the studies of He et al. [34] for hipO (*C. jejuni*) and LaGier et al. [35] for glyA (*C. coli*). The ntb2 gene fragment from Nicotiana tabacum (Anderson et al. [36]) was integrated as IAC. Primers and probes for ddPCR are characterized in Table 1.

The published triplex v-qPCR method [18], which is combining the 16S rRNA-based quantification of thermophilic *Campylobacter* with an Internal Standard Process Control (ISPC), as well as an IAC, was compared to our v-ddPCR.

2.7. Optimization of ddPCR

Absolute quantification using ddPCR was done using QX100™ Droplet Digital PCR System (Bio-Rad Laboratories, Hercules, CA, USA). The multiplex ddPCR validated here consisted of two detection systems in the FAM channel for *C. jejuni* and *C. coli*, and the ntb2 assay as an inhibition control in the HEX channel. The ddPCR reaction mix consisted of 1× ddPCR Supermix for Probes (with dUTP) (Bio-Rad Laboratories).

Table 1. Nucleotide sequence of oligonucleotides used in ddPCR.

Assay	Primer/Probe	Sequence 5′→3′	Amplicon Size [bp]	Final Concentration in ddPCR [nM]	Reference
C. jejuni (target gene *hipO* [1])	hipO-fw	TGCACCAGTGACTATGAATAACGA	124 (according to Acc. No. NC_002163.1)	350	He et al. [34]
	hipO-re	TCCAAAATCCTCACTTGCCATT		350	
	hipO-p	FAM [4]—TTGCAACCT*CACTAGCAAAATCCACAGCT—IABkFQ [6,7]		200	
C. coli (target gene *glyA* [2])	glyA-fw	CATATTGTAAAACCAAAGCTTATCGTG	133 (according to Acc. No. AF136494.1)	350	LaGier et al. [35]
	glyA-re	AGTCCAGCAATGTGTGCAATG		350	
	glyA-p	FAM [4]—TAAGCTCCA*ACTTCATCCGCAATCTCTCTAAATTT—IABkFQ [6,7]		200	
Internal PCR control (target gene ntb2 [3])	IPC-ntb2-fw	ACCACAATGCCAGAGTGACAAC	125	350	Anderson et al. [36]
	IPC-ntb2-re	TACCTGGTCTCCAGCTTTCAGTT		350	
	IPC-ntb2 probe	HEX [5]—CACGCGCAT*GAAGTTAGGGGACCA—IABkFQ [6,7]		200	

[1] hippurate hydrolase gene; [2] serine hydroxymethyltransferase gene; [3] methyltransferase gene of *Nicotiana tabacum*; [4] FAM: 6-carboxyfluorescein, [5] HEX: hexachlorofluorescein; [6] IABkFQ: 3′ Iowa Black® FQ (quencher); [7] = ZEN™ (internal quencher).

The primer and the probe concentrations were optimized to achieve an optimal fluorescence signal for all three primer–probe systems. The primer–probe systems met the quality criteria of the MIQE guidelines [37] with 100% efficiency and a coefficient of determination of $r^2 \geq 0.98$ (data not shown). Final concentrations of primers (TIB MOLBIOL, Berlin, Germany) and probes (IDT, Coralville, IA, USA) are outlined in Table 1. As an internal amplification control, 50 copies of ntb2 target were added. The reaction mix was filled with PCR-grade water to 15 µL. A 5 µL sample DNA was added and mixed thoroughly.

For droplet generation, the whole reaction volume (20 µL), as well as 70 µL droplet generation oil were transferred into a DG8™ droplet generator cartridge (Bio-Rad Laboratories) according to the manufacturer's instructions. Droplets were produced using a QX100™ Droplet Generator (Bio-Rad Laboratories). A total of 40 µL of the generated droplets (Water-oil emulsion) were subsequently transferred into a 96-well plate, closed with a pierceable foil heat seal (Bio-Rad Laboratories), and sealed using a PX1™ plate sealer (Bio-Rad Laboratories) at 180 °C for 5 s. An endpoint PCR was performed on a T1000 Touch Thermal Cycler (Bio-Rad Laboratories), using the following cycling protocol: enzyme activation at 95 °C for 10 min, followed by 45 cycles of 94 °C for 30 s and 56 °C for 1 min, followed by a droplet stabilization step at 98 °C for 10 min and a final hold step at 4 °C. The optimal annealing temperature of 56 °C was determined by a gradient PCR experiment in which an annealing temperature gradient between 55 °C and 60 °C was applied.

Droplet measurement was performed on a QX100™ Droplet Reader (Bio-Rad Laboratories). The QuantaSoft™ Software Version 1.7.4.0917 (Bio-Rad Laboratories) was used for data analysis.

2.8. qPCR

The qPCR method using the 16S rRNA gene for quantification of living thermophilic *Campylobacter* spp., including *C. sputorum* as ISPC and ntb2 gene as IAC as well as the data analysis, was performed according to Stingl et al. [18].

2.9. Live/Dead Differentiation of Campylobacter in Meat Rinse Samples

For live/dead differentiation of *Campylobacter* using ddPCR or qPCR, meat rinses were divided into two aliquots, each with a volume of 1 mL. One of these aliquots was treated with PMA dye (Biotium, Fremont, CA, USA) at a final concentration of 50 µM. This aliquot was incubated for 15 min at 700 rpm at 30 °C in the dark by covering the incubator with aluminum foil. Subsequently, it was transferred to phAST Blue equipment (genIUL instruments, Terrassa, Spain) for 15 min light exposure. This procedure was performed to cross-link PMA to DNA in dead bacteria at room temperature. Following that, both the PMA treated and the untreated aliquots were centrifuged at 16.000× *g* for 5 min at 4 °C. The supernatant was discarded and the cell pellet was either directly subjected to DNA extraction or frozen at −20 °C until DNA extraction.

The total amount of *Campylobacter*, including live- and dead-cells, was determined from the aliquot without PMA treatment, and it served as control for the PMA efficiency in reducing the dead cell signal. Only the results from PMA treatment were directly comparable to the results from the classical microbiological method. This enabled the quantification of the number of viable *Campylobacter* cells. For every sample, the dilution with the highest viable *Campylobacter* count was considered as the final result, considering different factors, including initial dilution of the sample in peptone water, elution volume for DNA extraction, total reaction volume, and the DNA volume used in ddPCR. All results were converted into $\log_{10}$. *Campylobacter* positive results were interpreted in two different categories: above the limit of PHC ($\log_{10}$ 3.0 live counts/mL) and below the limit of PHC.

2.10. Microbiological Reference Method

The classical microbiological method was carried out according to ISO 10272-2:2017 [7]. A 1 mL meat rinse was spread onto 3 mCCD agar plates (modified Charcoal-Cefoperazone-

Deoxycholate Agar) (Merck KGaA, Darmstadt, Germany), and incubated at 42 °C for 44 h ± 4 h under microaerobic conditions. All typical *Campylobacter* colonies were counted and MALDI Biotyper (Bruker Daltonics, Bremen, Germany) was used according to Huber et al. [32] to identify and to exclude doubtful colonies.

2.11. Determination of $LOD_{95\%}$

To determine the lowest copy number, still detectable with a 95% confidence interval ($LOD_{95\%}$) in the duplex v-ddPCR method, two distinct serial dilutions of the target DNA from *C. jejuni* and *C. coli* were prepared at 6 low copy number levels (4, 2, 1, 0.4, 0.2 and 0.02 copies/µL). Sonicated salmon sperm DNA solution (10 ng/µL) (Thermo Fisher Scientific Inc.) was used for dilution of both species to maintain the stability of genomic DNA. Each dilution level was measured using a duplex v-ddPCR in 12 independent technical replicates. The probability of detection (POD curve) and $LOD_{95\%}$ was computed via a web service provided by QuoData (QuoData Web Service [38]) according to BVL guidelines [39].

2.12. Precision—Relative Repeatability Standard Deviation (RSDr)

The relative standard deviation of repeatability (RSDr) was calculated over the whole dynamic range of the v-ddPCR assay and under repeatability conditions, according to the JRC Technical Report [40]. A total of 5 technical replicates of the target DNA of *C. jejuni* and *C. coli* were measured at 4 different concentrations (5, 50, 500, and 2000 cp/µL) over 5 days. The Excel statistical technique One-way ANOVA (Analysis of Variance, Single-factor) was used to calculate the RSDr for each dataset of 25 test results. These analyses estimate the significant differences between group means.

2.13. Determination of LOQ

The limit of quantification (LOQ) is the lowest copy number concentration in a sample that can be reliably quantified with an uncertainty considered acceptable for the intended use of the method (JRC Technical Report [40]). The LOQ was assessed by applying a linear model to RSDr results (refer to Section 2.12).

2.14. Selectivity

The hipO and glyA primer sets were evaluated with pure cultures of reference strains and well-characterized isolates (refer to Section 2.1) for their exclusivity and inclusivity. As stated in Section 6.1.5 of ISO 16140-2:2016 [30], at least 50 pure target strains and 30 pure non-target strains should be included in the testing procedure. In our study, all DNA concentrations were adjusted to 10 pg/µL. Inclusivity testing was performed on the DNA isolates of 50 *C. jejuni* and 41 *C. coli* in a duplex v-ddPCR. For exclusivity testing, 31 non-target DNAs, consisting of 5 reference strains from the family Campylobacteraceae (2 *C. upsaliensis*, 1 *C. lari*, 1 *C. lari concheus*, and 1 *C. perloridis*), and 26 reference strains from the non-Campylobacteraceae family were tested in a duplex v-ddPCR.

2.15. Method Comparison Study According to ISO 16140-2:2016

The accuracy and relative trueness studies were performed independently for *C. jejuni* and *C. coli* according to ISO 16140-2:2016 [30] by comparing the ddPCR against the microbiological reference method.

Live and dead cell standards of *C. jejuni* and *C. coli* (refer to Section 2.2) were spiked to 3 mL *Campylobacter* free meat rinse (refer to Section 2.3). Following a ddPCR data analysis, statistical calculations were performed according to the principles explained in ISO 16140-2:2016 [30], Section 6.1.2. for the relative trueness and Section 6.1.3. for the accuracy.

2.15.1. Accuracy

To cover the whole range of contamination, six chicken neck skin rinses were artificially spiked with *C. jejuni* or *C. coli* live/dead cell standards at three different levels (low A1,

A2; medium A3, A4; and high A5, A6, see Table 2). For all six samples of *C. jejuni* and *C. coli*, five biological replicates (e.g., for low-level A1–1, A1–2, A1–3, A1–4, and A1–5) were prepared and analyzed in a duplex v-ddPCR against the classical microbiological method.

Table 2. Spiking plan for the accuracy study. For each sample (A1 to A6), 3 mL chicken neck skin rinse were spiked with live- and dead-cell standards of *C. jejuni* and *C. coli*, respectively, in five biological replicates.

Sample	Level and Organic Matter Content of Meat Rinse/mL	Bacterial Contamination Level	*C. jejuni*/ *C. coli* Live CFU/mL	*C. jejuni*/ *C. coli* Dead Cells/mL	Biological Replicates
A1	Low/12 mg	low	200	2000	5
A2	Low/12 mg	low	500	5000	5
A3	Low/12 mg	medium	1000	10,000	5
A4	Low/12 mg	medium	2000	20,000	5
A5	Low/12 mg	high	5000	50,000	5
A6	Low/12 mg	high	10,000	100,000	5

2.15.2. Relative Trueness

To monitor the robustness against matrix variation, the trueness study was conducted on three different raw meat matrices, including chicken neck skin from the slaughterhouse, chicken breast, and turkey skin from retail shops. For this purpose, all three meat matrices containing low organic matrix (LM) were artificially spiked with five levels of *C. jejuni* and *C. coli* live/dead cell standards (T1-LM to T5-LM). Furthermore, all three meat items containing a medium organic matrix (MM) were artificially spiked with two bacterial levels (T2-MM and T5-MM) for comparison between two different matrix effects (Table 3). All samples were tested in a ddPCR against the classical microbiological method.

Table 3. Spiking plan for the trueness study. For each sample, 3 mL each of chicken neck skin, chicken breast, and turkey skin meat rinse were artificially contaminated with live- and dead-cell standards of *C. jejuni* and *C. coli*, respectively.

Sample	Matrices	Level and Organic Matter Content of Meat Rinse/mL	Bacterial Contamination Level	*C. jejuni*/ *C. coli* Live CFU/mL	*C. jejuni*/ *C. coli* Dead Cells/mL	Biological Replicate
T1-LM	Chicken neck skin/Chicken breast/Turkey skin	Low/8 mg	low	500	5000	1
T2-LM		Low/8 mg	low	1000	10,000	1
T2-MM		medium/37–48 mg	low	1000	10,000	1
T3-LM		Low/8 mg	medium	2000	20,000	1
T4-LM		Low/8 mg	medium	5000	50,000	1
T5-LM		Low/8 mg	high	20,000	200,000	1
T5-MM		medium/37–48 mg	high	20,000	200,000	1

2.16. Technical Measurement Uncertainty

The technical measurement uncertainty for the ddPCR method was determined according to the global approach (top-down approach) described in ISO 19036:2019 [31]. In order to ensure an efficient and a reliable estimation of in-house reproducibility, a factorial design was implemented. Seven factors were chosen to represent the range of conditions during routine testing (Table 4). In this design, measurements were performed in eight different runs, with each run corresponding to a combination of factor levels. Depending on the spiking level of the chicken neck skin with *C. jejuni* or *C. coli* live/dead cell standards, a total of five artificially contaminated samples (TU1 to TU5, Table 5) were analyzed for every run.

Table 4. Factorial design for the evaluation of technical uncertainty (7 factors).

	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6	Factor 7
	Day	Scientist	Mastermix	Time interval: DNA Extraction to generation of droplets	Time interval: Generation of droplets to PCR reaction	Time interval: PCR reaction to Droplet reading	Cartridge for generation of droplets
	day 1 vs. day 2	2 different Scientists	with vs. without dUTP	immediately vs. 3 nights freezing	immediately vs. after 30 min	immediately vs. overnight 4 °C	batch 1 (Lot. C000112961) vs. batch 2 (Lot. C000114859)
Run 1	day 1	1	with	immediately	immediately	immediately	batch 1
Run 2	day 1	1	with	3 nights	30 min	overnight	batch 2
Run 3	day 1	2	without	3 nights	immediately	immediately	batch 2
Run 4	day 1	2	without	immediately	30 min	overnight	batch 1
Run 5	day 2	2	with	3 nights	immediately	overnight	batch 1
Run 6	day 2	2	with	immediately	30 min	immediately	batch 2
Run 7	day 2	1	without	immediately	immediately	overnight	batch 2
Run 8	day 2	1	without	3 nights	30 min	immediately	batch 1

Table 5. Spiking plan for the technical measurement uncertainty study. For each sample, chicken neck skins (low organic matter content of meat rinse, 12 mg) were artificially contaminated with live and dead cell standards.

Sample	Bacterial Contamination Level	*C. jejuni* Live CFU/mL	*C. jejuni* Dead Cells/mL	*C. coli* Live CFU/mL	*C. coli* Dead Cells/mL	Total Live Cells/mL	Total Dead Cells/mL	Total Number of Spike for Eight Runs
TU1	low	150	750	50	250	200	1000	26
TU2	low	125	625	375	1875	500	2500	26
TU3	Medium	250	1250	750	3750	1000	5000	18
TU4	High	3750	18,750	1250	6250	5000	25,000	18
TU5	High	7500	37,500	2500	12,500	10,000	50,000	18

The low bacterial contamination samples (TU1 and TU2) were analyzed in three biological replicates for each of eight runs: 26 mL of chicken neck skin rinse were spiked (24 mL for ddPCR with PMA and 2 mL for the reference method). The medium (TU3) and high bacterial contamination (TU4 and TU5) were analyzed in two biological replicates for each of eight runs: 18 mL of chicken neck skin rinse were spiked (16 mL for ddPCR with PMA and 2 mL for the reference method). For the reference method, two biological replicates were analyzed immediately after spiking.

3. Results

3.1. Determination of $LOD_{95\%}$

The $LOD_{95\%}$ was determined with 0.837 cp/µL with a 95% confidence interval of [0.535, 1.309] for *C. jejuni* and 1.140 cp/µL and with a 95% confidence interval of [0.540, 2.000] for *C. coli*, which corresponds to 4.2 cp/reaction (0.6 $\log_{10}$ cp/reaction) for *C. jejuni* and 5.7 cp/reaction (0.8 $\log_{10}$ cp/reaction) chicken rinse for *C. coli*.

3.2. Precision and RSDr

The calculated RSDr decreased by increasing the copy number concentration and amounts to 28.1%, 17.1%, 7.5%, 4.9% (*C. jejuni*) and to 23.9%, 15.9%, 5.9%, 4.7% (*C. coli*) for 5, 50, 500, 2000 cp/µL, respectively. The RDSr values of each dataset were plotted against the copy number concentrations ($\log_{10}$) measured in a v-ddPCR. Figure 1a,b (solid-filled line, for one technical replicate) demonstrated a linear correlation y = ax + b with a coefficient of determination R^2 between 0.97 and 0.98. This linear regression model fits well with the observed data over the measured range of the v-ddPCR assay for both species.

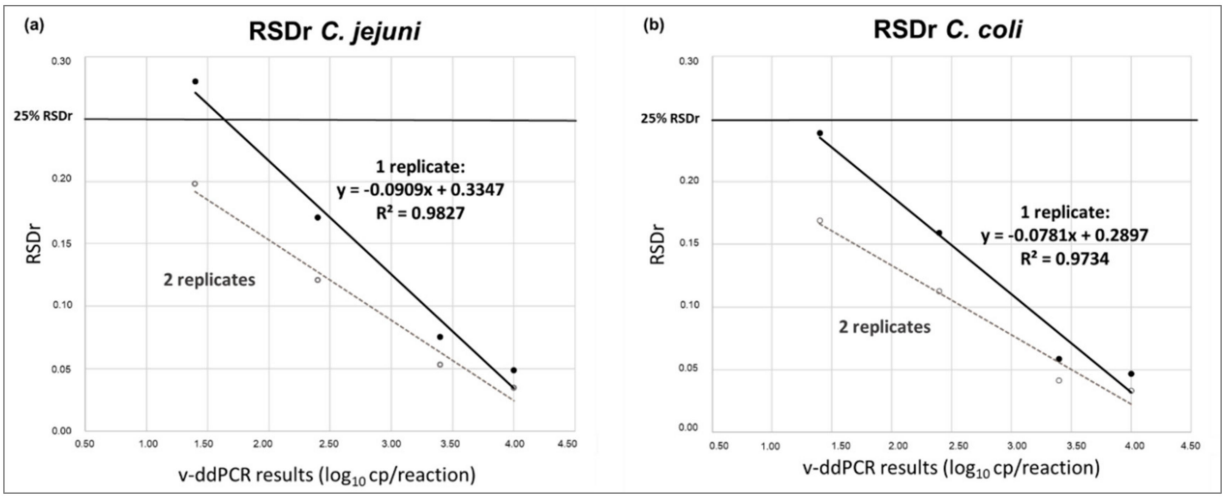

Figure 1. Relative standard deviation of repeatability (RSDr) calculated under repeatability conditions for (**a**) *C. jejuni* and (**b**) *C. coli*. Estimation of LOQ (RSDr value of 25%) for one tested replicate (solid-filled points and line) and for two replicates (non-filled points and dashed line).

3.3. Determination of LOQ

The linear regression (Figure 1a,b, solid-filled line for one replicate) was used to determine the LOQ at an RSDr of 25%. The LOQ value equals to 42 cp/reaction (1.6 $\log_{10}$ cp/reaction) for *C. jejuni* and 16 cp/reaction (1.2 $\log_{10}$ cp/reaction) for *C. coli* at RSDr of 25%. The addition of a second replicate in the ddPCR analysis reduced the RSDr, allowing a better detection of PHC (Figure 1a,b, dashed line).

3.4. Inclusivity and Exclusivity Study

All 50 *C. jejuni* and 41 *C. coli* DNAs were quantified between 3800 and 15,000 copies/µL, while no signal—except in IAC—was detected for the 31 non-target DNAs. Thus, the inclusivity and the exclusivity for hipO and glyA primer sets in duplex v-ddPCR successfully passed with 100%.

3.5. Method Comparison Study According to ISO 16140-2:2016

Accuracy and relative trueness studies were conducted on raw meat matrices, artificially spiked with different live and dead *Campylobacter* levels (1:10 ratio) and tested in duplex v-ddPCR with and without prior PMA treatment. The design used for the comparison studies and the subsequent evaluation was performed according to ISO 16140-2:2016(E) [30].

3.5.1. Accuracy Profile Study

Based on the central values (medians) of the ISO reference method X_i, and the v-ddPCR alternative method Y_i, the deviation of the alternative method from the reference method (absolute bias B_i) was calculated for all samples. No systematic bias was observed for *C. coli* samples, only a slight bias was noticed for *C. jejuni* samples, especially for A2 (+0.20 $\log_{10}$ counts/mL) and A3 (+0.27 $\log_{10}$ counts/mL). Additionally, the standard deviation of the alternative method s_{alt} was determined across all samples (0.083 $\log_{10}$ counts/mL for *C. jejuni* and 0.112 $\log_{10}$ counts/mL for *C. coli*). The standard deviation of the reference method s_{ref} equals to 0.068 $\log_{10}$ counts/mL. Taking into account the number of biological replicates (n = 5), the upper and the lower β-expectation tolerance interval β-ETI (Ui and Li) were calculated for each sample, and they lie within the acceptability limits ($-AL$ and $+AL$) of ± 0.50 $\log_{10}$ counts/mL. All relevant statistical results are provided in Tables 6 and 7. In accordance with ISO 16140-2:2016(E) [30], the alternative method is accepted as being equivalent to the reference method regarding the used raw chicken neck skin.

Table 6. Results of the accuracy profile study on chicken neck skin for *C. jejuni* [$\log_{10}$ live counts/mL].

Sample	Central Value Ref. Method X_i	Central Value Alt. Method Y_i	Absolute Bias B_i	Upper β-ETI Ui	Lower β-ETI Li	Upper AL $+AL$	Lower AL $-AL$
A1	2.25	2.34	0.09	0.21	−0.03	+0.50	−0.50
A2	2.55	2.75	0.20	0.32	0.08		
A3	2.83	3.09	0.27	0.39	0.15		
A4	3.18	3.30	0.12	0.24	0.00		
A5	3.66	3.71	0.05	0.17	−0.07		
A6	3.94	4.03	0.09	0.21	−0.03		

Standard deviation of the reference method s_{ref} 0.068 $\log_{10}$ live counts/mL. Standard deviation of the alternative method s_{alt} 0.083 $\log_{10}$ live counts/mL. β-ETI: β-expectation tolerance interval expected to cover 80% of the future measurements, AL: acceptability limit, given as a difference between reference and alternative methods and set at ±0.50 $\log_{10}$ live counts/mL (ISO 16140-2:2016(E) [30]).

Table 7. Results of the accuracy profile study on chicken neck skin for *C. coli* [$\log_{10}$ live counts/mL].

Sample	Central Value Ref. Method X_i	Central Value Alt. Method Y_i	Absolute Bias B_i	Upper β-ETI Ui	Lower β-ETI Li	Upper AL $+AL$	Lower AL $-AL$
A1	2.23	2.30	0.07	0.23	−0.09	+0.50	−0.50
A2	2.70	2.78	0.08	0.24	−0.08		
A3	3.03	3.04	0.02	0.18	−0.14		
A4	3.36	3.46	0.10	0.26	−0.06		
A5	3.76	3.77	0.00	0.16	−0.16		
A6	3.92	4.02	0.09	0.25	−0.07		

Standard deviation of the reference method s_{ref} 0.068 $\log_{10}$ live counts/mL. Standard deviation of the alternative method s_{alt} 0.112 $\log_{10}$ live counts/mL. β;-ETI: β-expectation tolerance interval expected to cover 80% of the future measurements, AL: acceptability limit, given as a difference between reference and alternative methods and set at ±0.50 $\log_{10}$ live counts/mL (ISO 16140-2:2016(E) [30]).

3.5.2. Relative Trueness Study

The scatter plot in Figure 2a for *C. jejuni* illustrates slightly higher $\log_{10}$ IPIU/mL values of the duplex v-ddPCR method for a low organic matrix (△○□) and slightly lower values for a medium organic matrix (▲●■) compared to the $\log_{10}$ CFU/mL values of the colony–count method. The scatter plot for *C. coli* (Figure 2b) revealed no significant difference between the microbiological reference method and the ddPCR alternative method since all data points except for two medium organic matrices of chicken neck skin (●) lie on the line of identity.

The Bland–Altman difference plot is a graphical method, describing agreements between two quantitative measurements. Figure 3 illustrates differences between two methods that are plotted against the means for each sample. The line of identity (difference of 0), the line of systematic deviation between both methods, as well as a 95% confidence interval for the upper and the lower limits of agreement [41] are additionally presented in the plot. All 42 spiked samples (low and medium organic matrix, 3 types of raw meat, 2 organisms *C. coli* and *C. jejuni*) are scattered all over the place, above and below the line of identity. The mean difference $\overline{D}$ between the alternative and the reference method is −0.02 $\log_{10}$ counts/mL close to zero. Based on this mean difference $\overline{D}$, the standard deviation of the differences (s_D = 0.29 $\log_{10}$ counts/mL) as well as the number of samples (n = 42), the limits of agreement were calculated according to ISO 16140-2:2016(E) [30] with a lower limit of −0.61 $\log_{10}$ counts per mL and an upper limit of 0.58 $\log_{10}$ counts/mL.

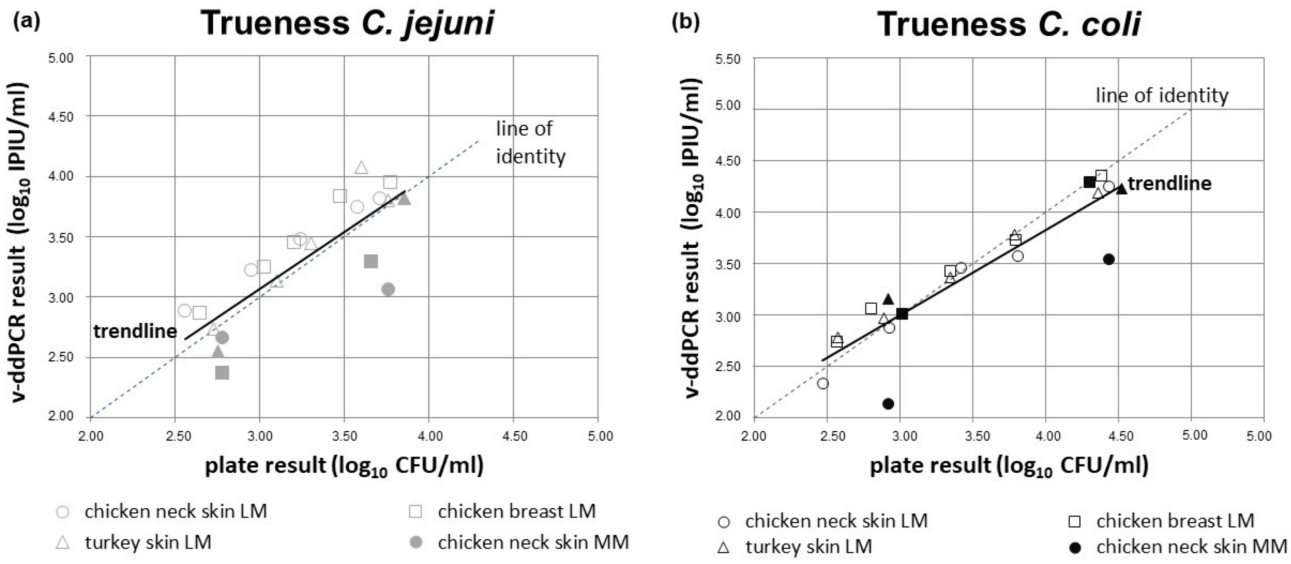

Figure 2. Scatter plot of alternative v-ddPCR method versus the microbiological reference method for determination of the relative trueness on three different types of raw meat matrices for (**a**) *C. jejuni* and (**b**) *C. coli*. LM: Low organic Matrix (represented by non-filled measurement points); MM: Medium organic Matrix (represented by solid-filled measurement points).

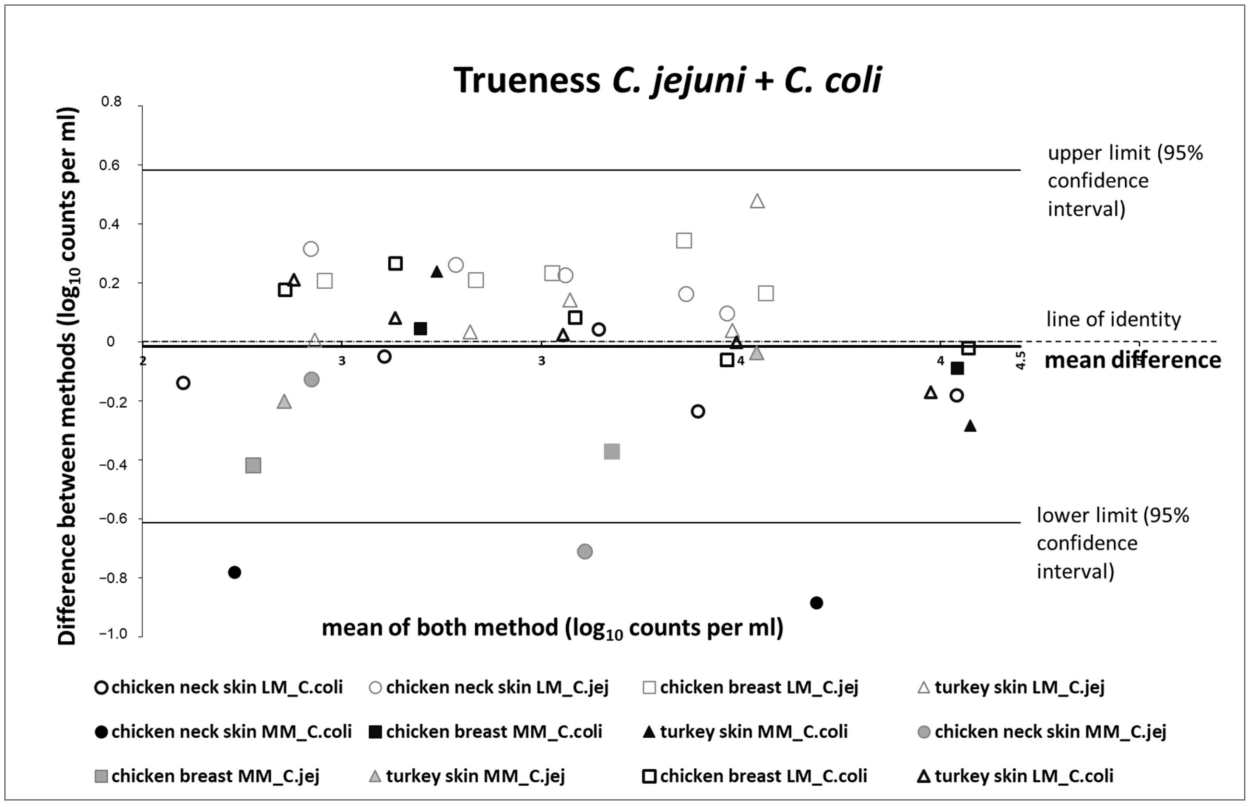

Figure 3. Bland–Altman difference plot for three different types of raw meat (*C. jejuni* and *C. coli*, low and medium organic matrix). LM: Low organic Matrix; MM: Medium organic Matrix, grey measurement points for *C. jejuni*, black measurement points for *C. coli*.

According to ISO 16140-2:2016(E) [30], it is expected that no more than 1 in 20 (5%) data values lie outside the upper and lower limits of agreement. For the data number between 41 and 60, not more than 3 data values are allowed to lie outside the limits. Our data show 3 outliers out of 42 samples falling beyond the limit of agreement. This suggests

that there is no consistent bias between both methods. The ddPCR provides equivalent results to the ISO standard method.

3.6. Technical Measurement Uncertainty

For the random effects, repeatability and run standard deviations were estimated (Table 8). The random variation between runs was negligible, i.e., total observed variation is explained by the factors and the repeatability component. For the factorial effects, the mean difference in log copies per droplet across the two factor levels was calculated (Table 8). For the two random components (run and repeatability), and for each factor both a constant and a proportional term were estimated.

Table 8. Absolute value of parameter effects. Significant effects (5%) are highlighted.

Type	Effect	Estimate	
		Constant Term	Proportional Term
Random	Repeatability	1.039×10^{-1}	5.699×10^{-2}
	Run	4.560×10^{-5}	2.301×10^{-5}
Factorial	Day	0.005	0.113
	Technician	0.237	0.054
	Supermix	0.270	0.055
	Cartridge	0.051	0.025
	Time interval: extraction-droplet	0.067	0.086
	Time interval: droplet-PCR	0.037	0.008
	Time interval: PCR-reading	0.160	0.087

As shown in Table 8, for all factors except two (cartridge and time interval: droplet-PCR), at least one of the two terms (constant or proportional) is statistically significant. In particular, the two factors—Technician and Supermix—display a considerable effect. Due to the magnitude of the effect of the factor Supermix, the in-house reproducibility precision was estimated separately for the two Supermixes. As a result, it was no longer possible to obtain estimates for all seven factors on the basis of the two separate (smaller) data sets (corresponding to the two Supermixes). Accordingly, in each of the two evaluations, only three factorial effects were estimated: day, cartridge, and time interval: extraction-droplet.

At all five contamination levels, very similar relative standard deviation (i.e., the ratio between standard deviation and the mean value, abbreviated as RSD) values were obtained for both random and factorial effects for both Supermixes. As illustrated in Tables 9 and 10, random variability shows a reverse pattern with the contamination level. Increasing the contamination level from 200 to 10,000 cells/mL was followed by a reduction in variability (78.5% to 23.3%) for the Supermix without dUTP. Similarly, for the Supermix with dUTP, the variability was reduced from 75.4% to 22.1%. The binomial (Poisson) component corresponds to the distributional uncertainty, and it is very large at low contamination levels (200 cells/mL: 58% for Supermix without dUTP and 55.1% for Supermix with dUTP). The recovery correction term corresponds to the statistical uncertainty of the estimation of the mean copy numbers at each contamination level. For further details regarding the statistical model and the meaning of the different components, the reader is referred to Uhlig et al. ([42], preprint]).

Table 9. Calculated RSD value for the technical uncertainty components—Supermix without dUTP.

Living Cells/mL Nominal Value	Day	Cartridge	Time Interval: Extraction to Droplet Generation	Factorial Effects	Recovery Correction	Repeat-Ability	Run	Binomial (Poisson)	Total	Total Minus Binomial
200	16.4%	5.7%	37.0%	42.2%	16.5%	28.0%	0.0%	58.0%	78.7%	53.3%
500	14.6%	5.2%	27.6%	32.4%	11.5%	22.7%	0.0%	36.7%	55.2%	41.2%
1000	13.4%	4.9%	20.9%	25.8%	8.3%	19.1%	0.0%	25.7%	42.0%	33.2%
5000	11.8%	4.5%	7.6%	14.8%	7.7%	13.5%	0.0%	12.1%	24.7%	21.5%
10,000	11.6%	4.5%	4.6%	13.3%	10.5%	12.9%	0.0%	9.4%	23.3%	21.3%

Table 10. Calculated RSD value for the technical uncertainty components—Supermix with dUTP.

Living Cells/mL Nominal Value	Day	Cartridge	Time Interval: Extraction to Droplet Generation	Factorial Effects	Recovery Correction	Repeat-Ability	Run	Binomial (Poisson)	Total	Total Minus Binomial
200	38.2%	6.9%	11.4%	41.2%	17.8%	25.2%	0.0%	55.1%	75.4%	51.5%
500	29.5%	5.3%	11.4%	32.6%	12.6%	19.7%	0.0%	34.8%	53.1%	40.1%
1000	23.6%	4.1%	11.4%	26.9%	9.0%	15.8%	0.0%	24.5%	40.7%	32.5%
5000	13.7%	1.5%	11.4%	18.1%	5.5%	8.9%	0.0%	11.4%	23.8%	20.9%
10,000	12.4%	1.0%	11.4%	17.0%	7.8%	7.9%	0.0%	8.6%	22.1%	20.3%

The total technical uncertainty for both supermixes provided in Tables 9 and 10 correspond to ddPCR analysis with one technical replicate. Due to the large technical uncertainty, it was agreed that two technical replicates (duplicate determination in ddPCR) should be performed in routine testing. The corresponding uncertainties are provided in Table 11 for the Supermix with dUTP. For target copy numbers between 200 and 10,000 cells/mL, the technical uncertainty ranges between 0.26 to 0.08 $\log_{10}$ copies.

Table 11. Obtained technical uncertainty for Supermix with dUTP.

Living Cells/mL Nominal Value	Technical Uncertainty (One Replicate) in %RSD	Technical Uncertainty (One Replicate) in $\log_{10}$	Technical Uncertainty (Two Replicates) in %RSD	Technical Uncertainty (Two Replicates) in $\log_{10}$
200	75.4	0.32	60.8	0.26
500	53.1	0.23	44.1	0.19
1000	40.7	0.17	34.5	0.15
5000	23.8	0.10	21.1	0.09
10,000	22.1	0.09	19.7	0.08

3.7. Performance of v-ddPCR on Routine and Retail Samples

The quantification of target DNA in ddPCR (copy number concentration) is based on an absolute count of PCR positive droplets (with PCR amplification of the target gene) and PCR negative droplets (without PCR amplification of the target gene). As advised in Huggett [43] and the Digital MIQE Guidelines [37,44], quality controls were integrated in each ddPCR run and checked before subsequent analysis of samples results. A no template control (NTC) serves as a control for extraneous nucleic acid contamination of this PCR, and it monitor false-positive reactions; the extraction control (EC) excludes contamination during DNA extraction; and internal amplification control (IAC with 50 copies/µL) helps to detect PCR inhibitors co-isolated from the matrix, and it confirms the negative results of the sample. Moreover, positive controls with specific target DNA of reference strains

C. jejuni and *C. coli* (10 pg/µL ≙ 9000–13,000 cp/µL) allow for checking if the ddPCR worked correctly. Finally, a low number of droplets measured (<10,000 cp per 20 µL PCR) as well as the lack of negative droplets are also criteria to exclude subsequent analysis of samples results.

3.7.1. Routine Samples

A total of 19 chicken neck skins (NS) from Bavarian slaughterhouses (1:10 and 1:2 diluted), as well as 13 chicken breast meat (BM) from Bavarian supermarkets (1:10 diluted) were investigated for routine analysis. All samples were analyzed by three methods, with the exception of three samples (NS_02, 03 & 04) which were not examined by qPCR analysis.

Out of nineteen slaughterhouse chicken neck skin samples (Figure 4), solely one sample (NS-12) was analyzed negative with all methods, nine crossed the PHC limit with all methods, and three were detected below the PHC limit with all methods. An inconsistency was observed for the remaining six chicken NS. For three of them, at least one method detected *Campylobacter* above the PHC limit: NS_19 and NS_35 were above PHC only in v-qPCR, NS_20 was above PHC only on agar plates, no positive droplet was detected in v-ddPCR for these three samples. Finally, at least one of method out of three detected *Campylobacter* below PHC for the last three chicken neck skins NS_13, NS_22, and NS_29.

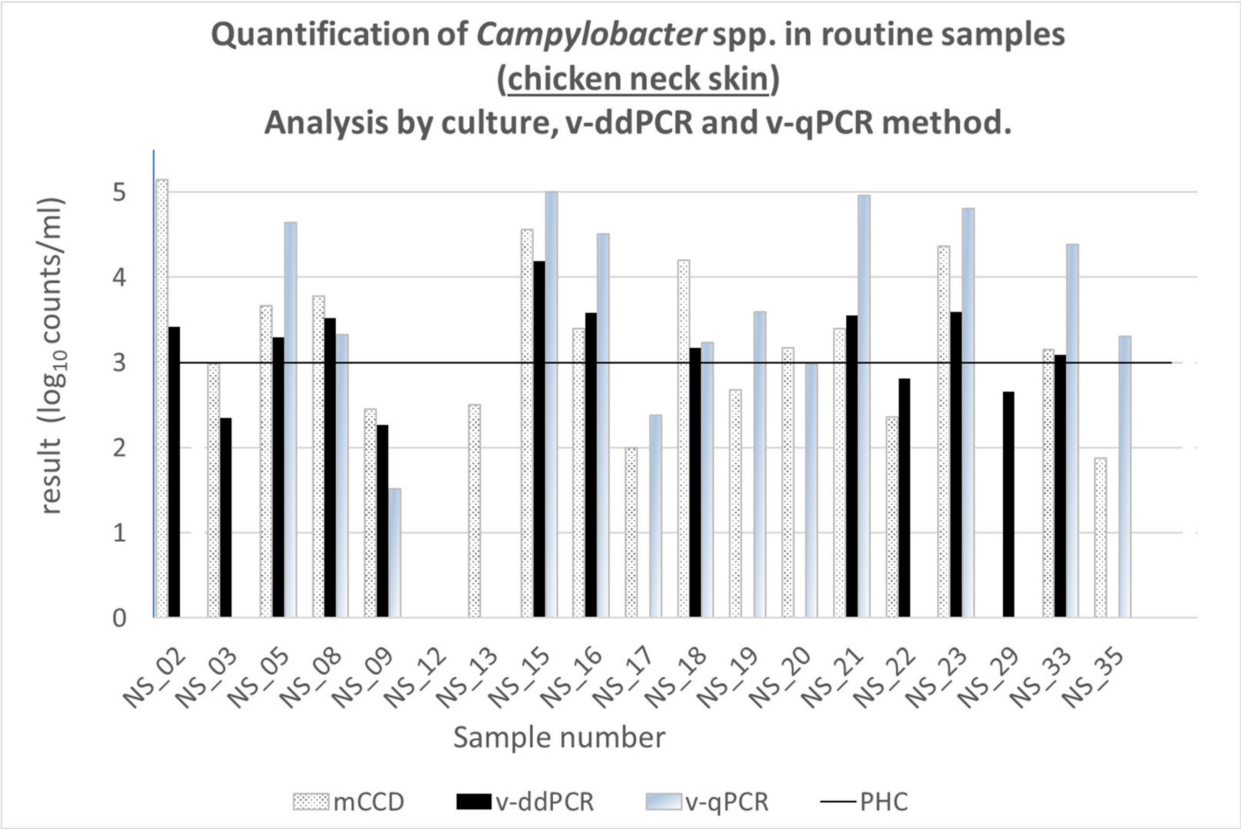

Figure 4. Analysis of LGL routine samples: chicken neck skin (NS) from slaughterhouse. Analysis in culture, v-ddPCR, and v-qPCR. The Process Hygiene Criterion (PHC: $\log_{10}$ 3.0) is marked with a black line.

Out of 13 breast meat samples (Figure 5), nine were negative with all three methods, and the other four did not reach the PHC limit. BM-30, BM-31, BM-32 were detected exclusively in the v-ddPCR method, whereas BM-11 was detected only on plates. No signal was detected in a v-qPCR analysis of all four samples.

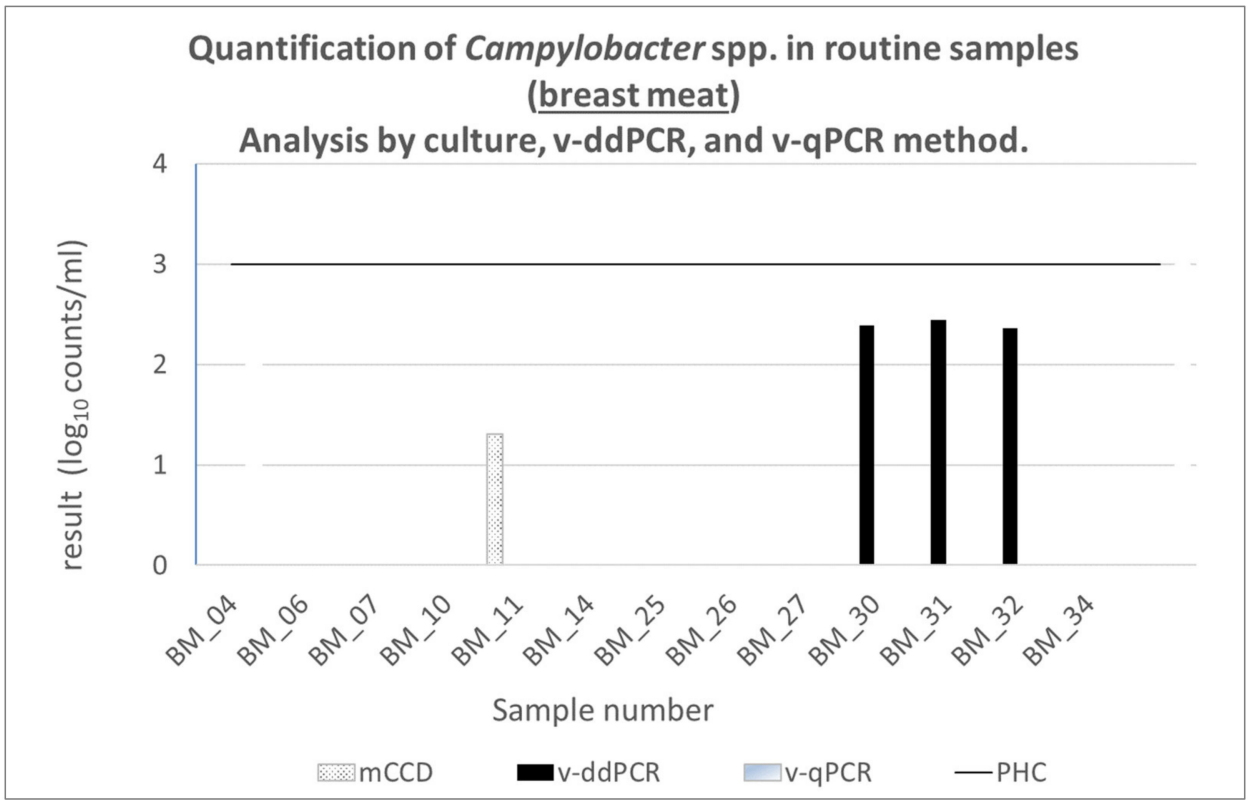

Figure 5. Analysis of LGL routine samples: chicken breast meat (BM) from retail. Analysis in culture, v-ddPCR and v-qPCR. The Process Hygiene Criterion (PHC: $\log_{10}$ 3.0) is marked with a black line.

Two routine samples (BM_27 and BM_34), as well as two retail samples (RS_21 and RS_24) were prone to matrix inhibition, as the IAC was not amplified in v-qPCR. The extracted DNA was further diluted to resolve the mentioned complication, and it was analyzed again in a v-qPCR. The absence of *Campylobacter* was confirmed in three samples (BM_27, BM_34, and RS_24) but RS_21 was *Campylobacter* positive in the v-qPCR. A signal in the IAC detection system showed that the ddPCR was not affected from this inhibition.

3.7.2. Retail Samples

Twenty-five raw chicken samples (RS) were collected from retail shops. As illustrated in Figure 6, 14 out of 25 RS, which build up 50% of the entire samples, were detected positive for *Campylobacter* with at least one method. Three of them were quantified above PHC (RS_03 and RS_15 in both PCR methods, RS_04 in a solely v-ddPCR method), indicating that the ISO standard method failed in the quantification of samples above PHC. The other 11 RS were detected below PHC limit based on at least one of the three methods.

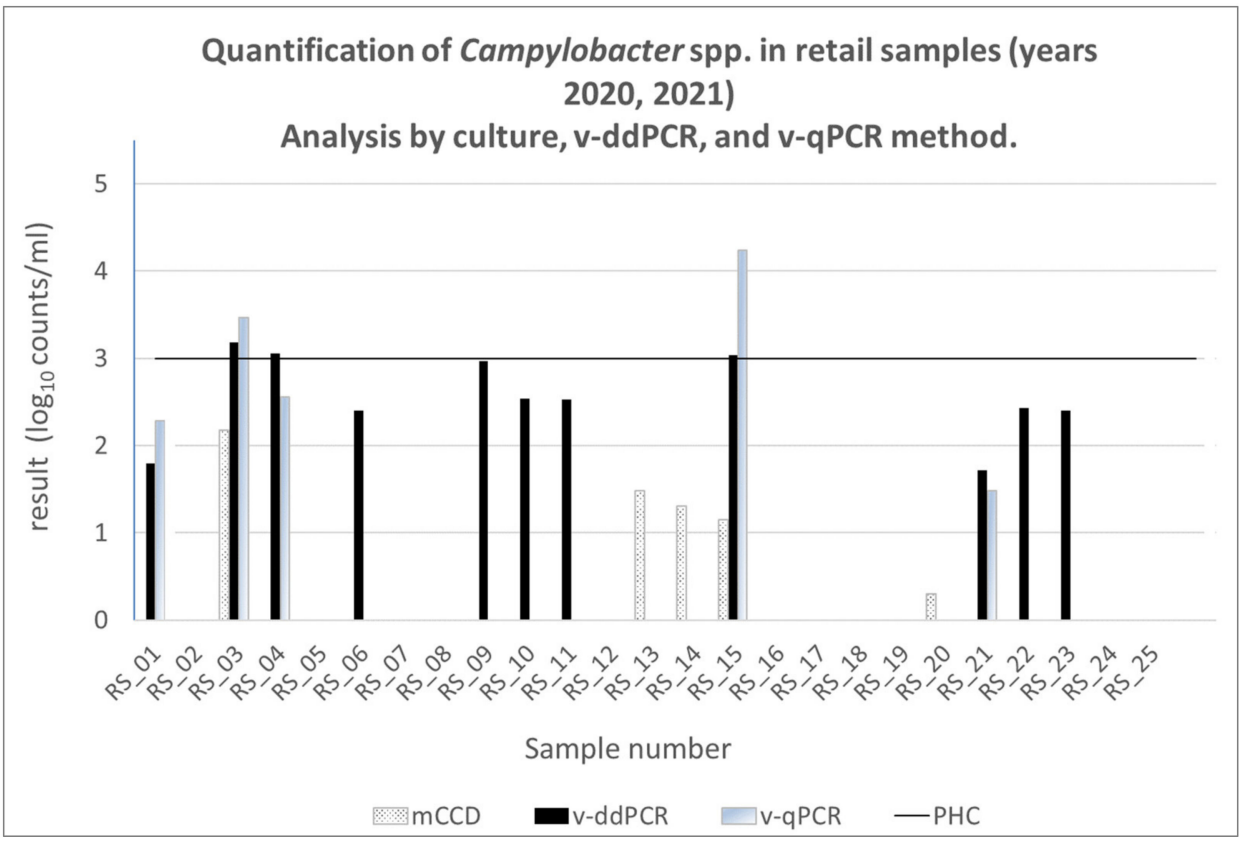

Figure 6. Analysis of different raw chicken (RS) meat samples from retail supermarket on culture, v-ddPCR and v-qPCR method. The Process Hygiene Criterion (PHC: $\log_{10}$ 3.0) is marked with a black line.

4. Discussion

C. jejuni and *C. coli* are the predominant *Campylobacter* species in poultry causing the most foodborne zoonosis and leading to a real impact on public health care. A review based on novel microbial risk assessment studies [5] like QMRA (Quantitative Microbial Risk Assessments) and based on CFU data shows the relationship between the prevalence of *Campylobacter* spp. in the poultry meat chain (broiler flocks, slaughterhouses, and consumers of poultry meat), and public health infection risk. The official ISO 10272-2:2017 method [7] used for the enumeration of *Campylobacter* spp. in poultry meat is unable to cultivate all infectious bacteria (IPIU), including CFU and VBNC, and it requires a long (2 days) and laborious (microaerobic atmosphere) incubation. Moreover, we observed the overgrowth of accompanying bacterial flora during the analysis of slaughterhouse samples, even on *Campylobacter*-selective mCCD agar plates, as well as samples with high levels of *Campylobacter* contamination make the microbiological enumeration of *Campylobacter* spp. unreliable for its use in routine analysis. A viability PMA-qPCR (v-qPCR) has been recently published [18], which overcomes these limitations, and it can be successfully applied for specific and sensitive quantification of *Campylobater* spp. in poultry. Digital PCR, the third generation of PCR technology, offers further advantages, such as an absolute quantification without an external calibration curve. Therefore, the final result is not influenced by the standard setting. In addition, due to the enrichment effect of the target of interest in the interfering background, ddPCR is highly tolerant to PCR inhibitors [28,45,46].

Applying a method comparison study according to ISO 16140-2:2016 [30], we internally validated a PMA-based duplex ddPCR method (v-ddPCR) against the official ISO 10272-2:2017 method [7] in order to quantify viable *C. jejuni* and *C. coli* in poultry meat of slaughterhouse and retail samples. The method was supposed to be practicable and cost-effective for routine analysis. On average, no consistent bias was observed between both

methods during the whole trueness study. Likewise, no detectable systematic bias was observed during the accuracy study for *C. coli*, in contrast, a slight positive bias (v-ddPCR against reference method) was noticed for *C. jejuni*. Finally, all criteria from ISO 16140-2:2016(E) [30] were fulfilled for both accuracy and relative trueness validation.

The Process Hygiene Criterion of maximum 1000 CFU *Campylobacter*/g chicken skin (1:10 dilution corresponds to 6.7 cp/reaction (0.8 $\log_{10}$ cp/reaction) and 1:2 dilution corresponds to 33 cp/reaction (1.5 $\log_{10}$ cp/reaction)) can be reliably detected with ddPCR, as the limit of detection is 4.2 cp/reaction (0.6 $\log_{10}$ cp/reaction) for *C. jejuni* and 5.7 cp/reaction (0.8 $\log_{10}$ cp/reaction) for *C. coli*. The evaluation of the limit of quantification (LOQ) was based on a precision experiment, and it reached 42 cp/reaction (1.6 $\log_{10}$ (cp/reaction) for *C. jejuni* and 16 cp/reaction (1.2 $\log_{10}$ (cp/reaction) for *C. coli* at RSDr 25%. We recommend, as reported by Strain [22], to increase the number of technical replicates analyzed in ddPCR so that the LOQ can be significantly decreased and the PHC can be reliably quantified (see Figure 1a,b). Likewise, the range of the technical uncertainty reaches a better performance if two technical replicates are included in the analyses (see Table 11). Furthermore, based on the uncertainty data we believe that a higher repeatability and reproducibility (in particular: lower factorial effects) can be achieved by better standardization of the method. Additionally, Kosir et al. [46] showed the critical impact of droplet volume variability in ddPCR on the accuracy of the absolute quantification.

The applicability of our v-ddPCR to quantify *Campylobacter* was investigated on natural contaminated routine samples from LGL, comprising chicken neck skin from slaughterhouses as well as breast meat and naturally contaminated retail samples, purchased in Bavarian supermarkets. The samples were analyzed in parallel with the reference method ISO 10272-2:2017 [7] and v-qPCR [18]. Huggett et al. [43] pointed out the characteristic and the unique potential of ddPCR technology to provide an absolute quantitative value of a specific target, but meanwhile there was a difficulty in verifying against other molecular methods. The used v-qPCR method relies on a calibration curve, delivering a quantitative value and thus confirming the accuracy of our ddPCR results when analyzing naturally contaminated samples. Solely, chicken neck skin samples from slaughterhouses (around 50% of analyzed samples) were quantified above PHC (3.0 $\log_{10}$ CFU/g chicken meat) on the reference plate count method; all breast meat and retail samples were detected under PHC on mCCD plates. The EU Regulation No 2017/1495 [6] already highlighted the existence of different significant contamination levels between neck skin and breast skin samples. Chicken neck skin samples from slaughterhouses have been shown to be much more contaminated than breast meat samples (skin-free) or retail samples from the supermarkets that are usually exposed to a long and stressful cooling of the refrigerated counter [19]. Except for NS-20, the plate results of chicken neck skin were confirmed with both alternative PCR methods, which revealed a good correlation of both PCR methods with the official plate count method for the slaughterhouse samples. For two neck skin samples, NS-19 and NS-35, *Campylobacter* could be quantified above the PHC only in a v-qPCR, and it remained undetected by the plate count method, as well as in the v-ddPCR. It cannot be excluded that another thermophilic *Campylobacter* spp. (e.g., *C. lari*) is involved, which could not be detected in a v-ddPCR method, which used other detection systems as v-qPCR and only detects *C. jejuni* and *C. coli*. This may also be related to the sensitivity of the different detection systems. While v-ddPCR is based on specific single-copy genes for *C. jejuni* and *C. coli*, three target copies of the 16S rRNA gene per chromosomal copy in *Campylobacter* spp. may be detected in v-qPCR [18].

Out of twenty-five retail samples, two samples—RS-03 and RS-15—were quantified above the PHC limit for both molecular methods; one additional sample, RS-04, was quantified above PHC only in v-ddPCR. The ISO standard method failed to quantify these three samples above PHC. *Campylobacter* may have lost its cultivability due to oxygen or cold stress during the 4 °C storage in supermarkets. These potentially infectious *Campylobacter* cells in the VBNC stage could not be microbiologically detected as CFU, so the level of contamination of poultry meat is clearly underestimated [47], and it represents a signif-

icant threat to human health. Hence, the current duplex v-ddPCR assay can be helpful for risk assessment of *Campylobacter* in broiler meat, especially for the previously cooled retail samples.

Finally, although we observed an acceptable correlation for quantifying *Campylobacter* between the three applied methods, none of the tested methods was successful in detecting all the *Campylobacter* positive samples beyond the PHC. Independent of the PHC, v-ddPCR could detect more *Campylobacter* positive samples (49%) than the other methods (v-qPCR 33%, plate count 40%) among the total number of samples. Worldwide, scientists have reported a higher sensitivity of ddPCR compared to qPCR [29,45]. The ddPCR partitions every sample into thousands of droplets, and it performs independent end-point PCR amplification on each droplet. Therefore, it enables the detection and the quantification of very low amounts of DNA copies. Additionally, based on the micro-dilutions that are carried out within each droplet, ddPCR is less affected by the interference of PCR-inhibitors from food matrices [28,48]. In our study, four routine samples were prone to the inhibition of IAC in qPCR but the PCR-inhibitors did not affect ddPCR. Thus, we were able to confirm Huggett's statement (dMIQE Group [37,43]), stating that dPCRs are less prone to inhibition compared to qPCRs. However, internal positive controls are strongly recommended to be integrated into the ddPCR runs [43].

Compared to the official plate counting ISO method, the recently published v-qPCR [18] and the ddPCR allow an efficient and reliable quantification of viable *Campylobacter* in poultry samples. Despite different methodologies (absolute quantification in ddPCR, standard-setting in qPCR) and different *Campylobacter* detection systems (specific single-copy genes in ddPCR, three target copies of the 16S rRNA gene in v-qPCR), both molecular methods deliver comparable results. Detection systems with single-copy genes provide the advantage that one gene copy corresponds to one CFU. A V-qPCR [18] was combined with an ISPC (Internal Sample Process Control) to monitor the PMA efficiency for reducing dead cells and to correct the DNA loss happening during sample processing. In our method comparison study (accuracy and trueness), PMA efficiency was monitored on 102 artificially spiked samples (ratio of 1 live: 10 dead bacteria). As expected, the sample portion with a PMA treatment analysis was comparable with the ISO standard plate count method. The results that were obtained from the sample portion without PMA treatment represented number of *Campylobacter* spp. that was approximately 5 to 10 times higher when compared to the sample with PMA, which proves the efficiency of PMA in reducing the dead cell signal.

A limitation of ddPCR regarding the dynamic range is the loss of linearity for samples with a high level of contamination [22,28]. The calculation of the absolute quantities in ddPCR is based on the number of positive vs. negative partitions in a sample, using Poisson statistics [45]. Samples with abundant contamination (Bio-Rad 20,000-droplet system achieves approximately a 4 $\log_{10}$ range) have to be preliminarily diluted so that the number of targets per partition is within a range suitable for Poisson quantification, minimizing the partitioning error in ddPCR [48]. However, we did not encounter this problem during the analysis of our natural contaminated samples from the slaughterhouse or from the Bavarian supermarket, where the maximum contamination was approximately 4 $\log_{10}$. Thus, v-ddPCR is well suited to a reliable, accurate, and sensitive quantification of *Campylobacter* in poultry, and it offers a practicable, rapid, and cost-effective alternative method for routine analysis.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app12115315/s1, List S1. Strains or isolates used in this study; List S2. Artificially contaminated and naturally contaminated rinses.

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