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Challenging the "gold standard" of colony-forming units - Validation of a multiplex real-time PCR for quantification of viable *Campylobacter* spp. in meat rinses

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ABSTRACT

Campylobacter jejuni is the leading bacterial food-borne pathogen in Europe. Despite the accepted limits of cultural detection of the fastidious bacterium, the "gold standard" in food microbiology is still the determination of colony-forming units (CFU). As an alternative, a live/dead differentiating qPCR has been established, using propidium monoazide (PMA) as DNA-intercalating crosslink agent for inactivating DNA from dead, membrane-compromised cells. The PMA treatment was combined with the addition of an internal sample process control (ISPC), i.e. a known number of dead *C. sputorum* cells to the samples. The ISPC enables i), monitoring the effective reduction of dead cell signal by the light-activated DNA-intercalating dye PMA, and ii), compensation for potential DNA losses during processing. Here, we optimized the method for routine application and performed a full validation of the method according to ISO 16140-2:2016(E) for the quantification of live thermophilic *Campylobacter* spp. in meat rinses against the classical enumeration method ISO 10272-2:2017. In order to render the method applicable and cost-effective for practical application, the ISPC was lyophilized to be distributable to routine laboratories. In addition, a triplex qPCR was established to simultaneously quantify thermophilic *Campylobacter*, the ISPC and an internal amplification control (IAC). Its performance was statistically similar to the two duplex qPCRs up to a contamination level of 4.7 $\log_{10}$ *Campylobacter* per ml of meat rinse. The limit of quantification (LOQ) of the alternative method was around 20 genomic equivalents per PCR

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reaction, i.e. 2.3 $\log_{10}$ live *Campylobacter* per ml of sample. The alternative method passed a relative trueness study, confirming the robustness against different meat rinses, and displayed sufficient accuracy within the limits set in ISO 16140-2:2016(E). Finally, the method was validated in an interlaboratory ring trial, confirming that the alternative method was fit for purpose with a tendency of improved repeatability and reproducibility compared to the reference method for CFU determination.

Campylobacter served as a model organism, challenging CFU as "gold standard" and could help in guidance to the general acceptance of live/dead differentiating qPCR methods for the detection of food-borne pathogens.

1. Introduction

According to German surveillance data and consistent with European observations, campylobacteriosis is the most frequently reported bacterial food-borne illness in humans (EFSA, 2021). Although the ultimate goal is a reduction to zero of food-borne pathogens, quantitative microbiological risk assessment has become an increasingly important issue in predicting the infectious potential of a given food matrix or source.

Campylobacter is a thermophilic, fastidious, microaerobic pathogen, successfully colonizing the intestine of chickens and of warm-blooded animals. The bacterium is completely different from *Salmonella* in chicken colonization characteristics, commonly reaching 1000-fold higher concentrations in the chicken gut and spreading in a flock within a few days (Van Gerwe et al., 2005). Consequently, fecal contamination during slaughtering leads to significant contamination of the chicken product. The fraction of highly contaminated chicken products is suggested to be one of the main causes for infection of consumers, since *Campylobacter* does not thrive on food products due to its fastidious growth demands. Accordingly, in 2018, the EU commission established a quantitative hygiene criterion with a threshold of 1000 CFU thermophilic *Campylobacter* per g chicken skin (at slaughterhouse) (Reg_EU_2017/1495).

As generally accepted by food authorities, under favorable growth conditions a viable bacterial pathogen forms a colony on an agar plate. Consistently, the ISO 10272-2:2017 norm (ISO 10272-2:2017) quantifies colony forming units (CFU) of *Campylobacter* and is quite useful when applied to fresh samples (e. g. from the slaughterhouse or fresh fecal droppings at primary production), where the intestinal bacterium just exited its natural niche of proliferation. However, the microbiological method is suboptimal for diagnostics, once the fastidious bacterium has been subjected to stress conditions ceasing growth on laboratory agar plates and exerting high variation of growth on different media (Carrillo et al., 2014; Lanzl et al., 2020; Répérant et al., 2016). The phenomenon, that *Campylobacter* switches to a viable but non-culturable state (VBNC) has been previously observed and oxidative and cold stress were considered as triggers for this persistence state (Chaisowwong et al., 2012; Kim et al., 2015; Oh et al., 2015; Rollins and Colwell, 1986). German monitoring data indicated that quantitative detection of *Campylobacter* on poultry meat at retail is hampered by loss of *in vitro* culturability of the bacterial pathogen due to cold and oxygen stress (Stingl et al., 2015; Stingl et al., 2012). Whereas nearly 50% of all samples of fresh chicken meat at retail were positive after enrichment (and probable recovery of transiently inactive *Campylobacter*) less than 5% of these samples tested *Campylobacter*-positive by direct plating (quantitative method). Still, it is indisputable that contaminated chicken meat products are harmful to the consumer, since 20–30% of all human campylobacteriosis cases have been attributed to the direct consumption and/or handling of chicken products. Source attribution studies even hint to 50–80% of the cases attributable to the chicken reservoir with epidemiological data missing (EFSA, 2011), thus, the transmission route is currently uncertain for the additional cases. Real-time PCR detection systems for *Campylobacter* are available, which however, are not yet standardized and validated for live/dead discrimination of bacteria (Josefsen et al., 2010; Mayr et al., 2010). The principle of selective amplification of DNA from intact and putatively infectious units (IPIU), comprising CFU and viable but non-culturable (VBNC) cells, is the pre-incubation of samples with the intercalating dye propidium monoazide (PMA) (Fittipaldi et al., 2012; Nocker et al., 2006). Note that we rigorously use the term VBNC as "viable but non-culturable" bacteria for all non-growing bacteria under standard laboratory conditions for praxis relevance. Hence, we do not differentiate reversible and irreversible stages of VBNC cells, the latter putatively only reflecting our current insufficient knowledge of parameters for "awakening" from the VBNC stage (Wulsten et al., 2020). PMA is passively excluded from viable cells, while the reagent enters membrane-compromised dead cells, intercalates into cytoplasmic DNA and blocks this DNA from PCR amplification via covalent crosslinking upon light induction. Various studies showed that viable *Campylobacter* can be detected by application of a combination of PMA treatment and qPCR and demonstrate the need for a validated and rigorously controlled standardized viability qPCR (v-qPCR) method (Castro et al., 2018; Duarte et al., 2015; Josefsen et al., 2010; Lazou et al., 2019; Lv et al., 2019; Magajna and Schraft, 2015; Seliwiorstow et al., 2015). High correlations between CFU and PMA qPCR values were revealed for fresh *Campylobacter* cultures, while substantial differences in counts were observed after long-term incubation and biofilm formation of *C. jejuni* (Magajna and Schraft, 2015). Moreover, *Campylobacter* spp. exposed to raw milk rapidly lost their capacity to form CFU, whereby the v-qPCR quantified viable *Campylobacter* in high concentrations (Wulsten et al., 2020). Intriguingly, growth of these VBNCs was "reactivated" by incubation under extremely low oxygen levels of 1% in the presence of hydrogen. This emphasized reliable results obtained by the v-qPCR and a drastic underestimation of the survival of *Campylobacter* in raw milk by CFU determination.

We recently established an internal sample process control (ISPC) in order to overcome previous limits of the method, namely variable residual PCR signal of dead cells and partial DNA losses during sample preparation (Pacholewicz et al., 2019). However, the method was not yet applicable for practical diagnostic use due to several reasons. First, the ISPC had to be prepared fresh, hampering distribution to routine laboratories. Second, two aliquots of the sample were processed using a single defined (high) concentration of ISPC - one with and one without PMA treatment. Hence, the read-out of signal reduction by PMA and DNA losses were not obtained from the same sample aliquot, neglecting putative intra-sample variations. Third, two labor- and cost-intensive duplex qPCRs had been established, in order to avoid competition of the relatively high concentrated ISPC on the target amplification signals. Here we present the development of an optimized v-qPCR method for the quantification of thermophilic *Campylobacter* spp. for practical use and a full validation study according to (ISO 16140-2:2016 (E)). The v-qPCR method was validated against the current microbiological enumeration norm (ISO 10272-2:2017), showing improved performance, while being consistent with CFU determinations. Filling in the gaps for reliable quantitative detection of *Campylobacter* will allow in the future for improved risk assessment studies, more targeted intervention strategies, and an overall reduction in the transmission of the pathogen.

2. Materials and methods

2.1. Strains and growth conditions

C. jejuni NCTC 11168 (BfR-CA-13601) or DSM 4688 and *C. sputorum* DSM 5363 from −80 °C cryobank stocks (MAST Group Ltd., Bootle, UK) were cultured on Columbia blood agar (ColbA, Oxoid, Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 5% sheep blood (Oxoid) under microaerobic conditions (5% O_2, 10% CO_2, rest N_2). *C. jejuni* was initially cultured for 24 h at 41.5 ± 1 °C and *C. sputorum* for 48 h at 37 ± 1 °C. Both strains were twice subcultured for another 24 h and subsequently for 18–22 h at 41.5 °C ± 1 °C or 37 ± 1 °C, respectively. For microbiological enumeration serial dilutions of the cell suspensions were spread on modified charcoal cefoperazone deoxycholate agar (mCCDA, Merck & Co., Kenilworth, New Jersey, USA or Oxoid). In the following, mean temperature conditions without the upper and lower limit of ±1 °C are mentioned for ease of reading.

2.2. Production and stabilization of the internal sample process control (ISPC)

Inactivation of *C. sputorum* cells, which served as ISPC, was slightly modified as done previously (Pacholewicz et al., 2019). In short, 18–22 h sub-cultured *C. sputorum* DSM 5363 were resuspended in pre-warmed brain heart infusion broth (BHI; Oxoid) at initial $OD_{600nm} = 0.05$. The culture was incubated for around 10 h under 140 rpm at 37 °C in a jar, which had been twice evacuated by vacuum and refilled with a gas mixture of 5% O_2, 10% CO_2 and rest N_2. After the culture had reached an $OD_{600} \sim 0.8$, a cell amount corresponding to 100 ml of $OD_{600} = 0.5$ was harvested by centrifugation in two 50 ml tubes at 8000 ×*g* for 30 min at 4 °C. "Physiological cell death" was simulated by oxidative stress inactivation. For this purpose, cells were resuspended in 100 ml phosphate-buffered saline (PBS; 135 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) supplemented with 5% H_2O_2 at an OD_{600} of 0.5 and treated for 25 min at room temperature (RT). Cells were aliquoted in six 50 ml tubes and centrifuged at 8000 ×*g* for 20 min at 4 °C. The supernatant was discarded and dead cells were transferred to two 2 ml tubes by resuspension in PBS and centrifuged at 16,000 ×*g* for 5 min. Supernatant was removed, cells were resuspended in PBS and pooled into one tube. OD_{600} was measured from an appropriate dilution. Inactivation of cells was checked by absence of growth of a 10 µl loop of an undiluted cell suspension on ColbA, incubated for 48 h at 37 °C under microaerobic conditions.

Lyophilisation medium was prepared as follows: 10% trehalose (Sigma Aldrich, Steinheim, Germany) was dissolved in Bolton broth basis (Oxoid) and sterile-filtered. Subsequently 5% fetal calf serum (FBS; PAN-Biotech GmbH, Aidenbach, Germany) and 1% autoclaved cell-culture tested glycerol (Sigma Aldrich, Steinheim, Germany) was added. Dead cells were added to 200 ml lyophilisation medium at a concentration of $OD_{600} = 0.2$ (corresponding to 10^9 cells/ml). Cell suspension in lyophilisation medium was kept on ice, and aliquoted to 1 ml portions in lyophilisation glass vials under constant stirring. Filled glass vials were kept on cooling elements or in the fridge until placing into the lyophilisation machine. The lyophilisation program was as follows: Temperature was decreased to −35 °C within the first 2.5 h at atmospheric pressure. Subsequently, the pressure was reduced to 0.6 mbar within 1 min and further decreased to 0.2 mbar within 20 h at −35 °C. Maintaining 0.2 mbar, temperature was risen to −15 °C within the next 10 h and further to 0 °C within the following 10 h. Finally, the pressure was reduced to 0.08 mbar at 4 °C for 8 h before glass vials were automatically sealed with rubber plugs. Thereafter, the vacuum was released, glass vials further sealed with aluminium caps and stored at 4 °C (if not otherwise stated). Homogeneity was analysed using 10 vials, in duplicate for the calculation of inter-vial and intra-vial variance, essential for the evaluation of homogeneity. For this purpose, 10^6 cells were either treated with or without PMA (Biotium, Hayward, CA, USA). Genomic equivalents were measured by duplex qPCR, targeting *C. sputorum* and IAC control. Stability of the lyophilisates was assessed after storage at 4 °C, 25 °C and 37 °C over 18 months and DNA accessibility in dead *C. sputorum* cells was analysed using PMA titration (0, 0.1, 0.5, 1, 5, 10 and 50 µM PMA).

For use, the lyophilisate was resuspended in 1 ml of buffered peptone water (PW; 1% peptone, 0.5% NaCl, 0.35% di-sodium hydrogen phosphate, 0.15% potassium di-hydrogen phosphate, pH 7.2), mixed well and reconstituted for 10 min on ice. After subsequent mixing, aliquots of 50-100 µl were stored at -80 °C and remained stable for at least six months.

2.3. Live/dead differentiation of Campylobacter spp. in meat rinse samples

An overview of the workflow is given in Fig. 1 and detailed in the Suppl. Information 1. In short, two aliquots of 1 ml of meat rinse were used for the optimized v-qPCR including ISPC monitoring as well as live-dead differentiation on the basis of PMA. Thereby, one aliquot was processed without PMA treatment to retrieve the total amount of thermophilic *Campylobacter* spp. and one aliquot with PMA treatment to retrieve the number of viable thermophilic *Campylobacter* spp. cells. DNA was extracted using the GeneJet Genomic DNA extraction kit (Thermo Fisher Scientific Inc.) according to the manufacturers' protocol with slight modification as described in the Suppl. Information 1. A volume of 10 µl was analysed by qPCR. For quality assurance, a negative extraction control was included.

2.4. Sample analysis by v-qPCR

In principle, two DNA standard series of genomic DNA were used for the triplex v-qPCR. It was assumed that 1 ng of genomic DNA of *C. jejuni* NCTC 11168 (1.6 Mb) corresponds to 5.94×10^5 genomic copies (Krüger et al., 2014). As the *C. sputorum* standard is only used for relative quantitative normalization, the same calculations were applied for the ISPC DNA standard. For practical use, we provide a mastermix calculator Excel sheet (Suppl. Information 2 v-qPCR_Campy_mastermix_calculator), including information on primer and probes according to (Pacholewicz et al., 2019) and the DNA standard preparation. First, stabilized dried genomic DNA of *C. sputorum* (DNAstable protectant, Biomatrica, San Diego, California, USA) was dissolved in 10 ng/µl salmon sperm DNA (Thermo Fisher Scientific Inc.) and diluted in the same protecting DNA background to reach 5000, 500, 50, 20, 10 copies/10 µl assay volume (Sp1–5). As DNA standard for thermophilic *Campylobacter* spp. in the presence of 1000 copies of ISPC, a solution containing 10 ng/µl of salmon sperm DNA and 100 copies/µl genomic equivalent of *C. sputorum* was first prepared (Csput diluant). Using this solution, stabilized dried genomic DNA of *C. jejuni* NCTC 11168 was dissolved and diluted in order to reach 5000, 500, 50, 20, 10 copies/10 µl of *C. jejuni* genomic equivalents in the background of 1000 copies of ISPC per 10 µl (Cjs1-5). Both serial DNA standards were applied in duplicate in each real-time PCR run, in order to obtain appropriate standard curves for conversion of Ct values in $\log_{10}$ copies of genomic equivalents. The DNA standards were aliquoted and stored at −20 °C for single use of each aliquot. The duplex qPCR was performed as described previously (Pacholewicz. et al., 2019). Each qPCR run contained genomic standards (genomic DNA of *C. jejuni* NCTC 11168 or *C. sputorum* DSM 5363 (ISPC)), comprising five serial dilutions in order to reach 5000, 500, 50, 20, 10 genomic copies per reaction in duplicate. These genomic standards were as described for the triplex qPCR but the genomic standard for thermophilic *Campylobacter* spp. was lacking the *C. sputorum* (ISPC) background DNA (Cj1-5). For details see Suppl. Information 2 v-qPCR_Campy_mastermix_calculator).

2.5. Preparation of reference material for method validation

Live quantitative *C. jejuni* NCTC 11168 cryostocks were produced and quality controlled as published on our website (NRL for Campylobacter, 2021). During cryoconservation live *C. jejuni* (5.51 ± 0.08 log_{10} CFU/ml displayed on mCCDA, Merck) partially lost viability and for spiking it was assumed that those bacteria had an approximate "intrinsic" 1:10 ratio of live/dead cells. Dead *C. jejuni* DSM 4688 were produced as follows. Starting from a cryobank stock, bacteria were inoculated on ColbA (Oxoid) for 24 h at 37 °C and 5% O_2, 10% CO_2, rest N_2. After subculture for another 18 ± 2 h under the same conditions, prewarmed BHI was inoculated with OD_{600} of 0.05 and incubated described above at 37 °C under 140 rpm shaking. After 6–7 h, bacteria were harvested in early stationary growth phase (OD_{600} 0.6–1.2) by centrifugation at 8000 ×*g* for 20 min at RT. The supernatant was discarded, and the cell pellet resuspended in PBS supplemented with 5% H_2O_2 and incubated for 45 min at room temperature before centrifugation at 4 °C at 8000 ×*g* for 30 min. The supernatant was withdrawn and the dead bacteria were resuspended and transferred to 2 ml tubes, washed once in PBS, and centrifuged at 16,000 ×*g* for 5 min. Absence of CFU was checked by streaking one loop of H_2O_2-treated bacteria on ColbA and incubation at 41.5 °C for 48 h under microaerobic atmosphere. Finally, the cell suspension was added to precooled cryomedium (Bolton broth basis, 5% FBS, 10% trehalose, 10% glycerol) to reach a final OD_{600} of 0.2, corresponding to 10^9 bacterial counts per ml. While aliquoting 1 ml per cryovial, the cell suspension was continuously kept in a cool rack at temperatures ranging from 0 °C to 4 °C (Biozym Scientific GmbH, Hessisch Oldendorf, Germany). Subsequently, the vials were shock-frozen in liquid N_2 for at least 10 s and kept on dry ice before long-term storage at −80 °C.

Rinse matrices were prepared starting from different food types of raw meat matrices, i.e. *Campylobacter* negative chicken meat, chicken skin or from a mixture of fresh pork and beef minced meat. Absence of *Campylobacter* was assessed by CFU and qPCR after enrichment according to (ISO 10272-1:2017). Samples were weighted and PBS was added at various volumes ranging from 1:2 to 1:10 of v/w of the sample. The samples were either rinsed manually or homogenized using a stomacher (Seward Limited, Worthing, UK) for 1–2 min and BagPage+ filter bags with a pore size of 280 μM (Interscience Lab Inc., Woburn, MA, USA). These variations guaranteed the preparation of chicken rinses with different contents of organic material (i.e. weight of wet material after centrifugation), simulating native matrices, which laboratories may encounter during routine work.

2.6. Method comparison study

The method comparison study, conducted at the Bavarian Health and Food Safety Authority (LGL) and the German Federal Institute for Risk Assessment (BfR) was in-line with ISO 16140-2:2017(E), Section 6.1. and comprised of (i) a relative trueness study for testing the robustness of the method against matrix variations, (ii) an accuracy study and (iii) the determination of the LOQ. For all parts of the method comparison study, live *C. jejuni* cryovials were thawed at RT for 15 min, followed by a 30 min incubation on ice for optimal CFU recovery before spiking of the matrices. *C. jejuni* dead cells were thawed and used directly upon appropriate dilution in PW. Cryovials of *C. jejuni* cells were kept on ice before spiking the matrices.

For the relative trueness study 3 ml rinse aliquots of 15 different matrices with an organic matter ranging from 4 to 76 mg/ml were spiked with various ratios of live and dead *C. jejuni* according to Suppl. Table 1. The v-qPCR was performed on two 1 ml aliquots with and without PMA treatment (see Section 2.3). After DNA extraction (Section

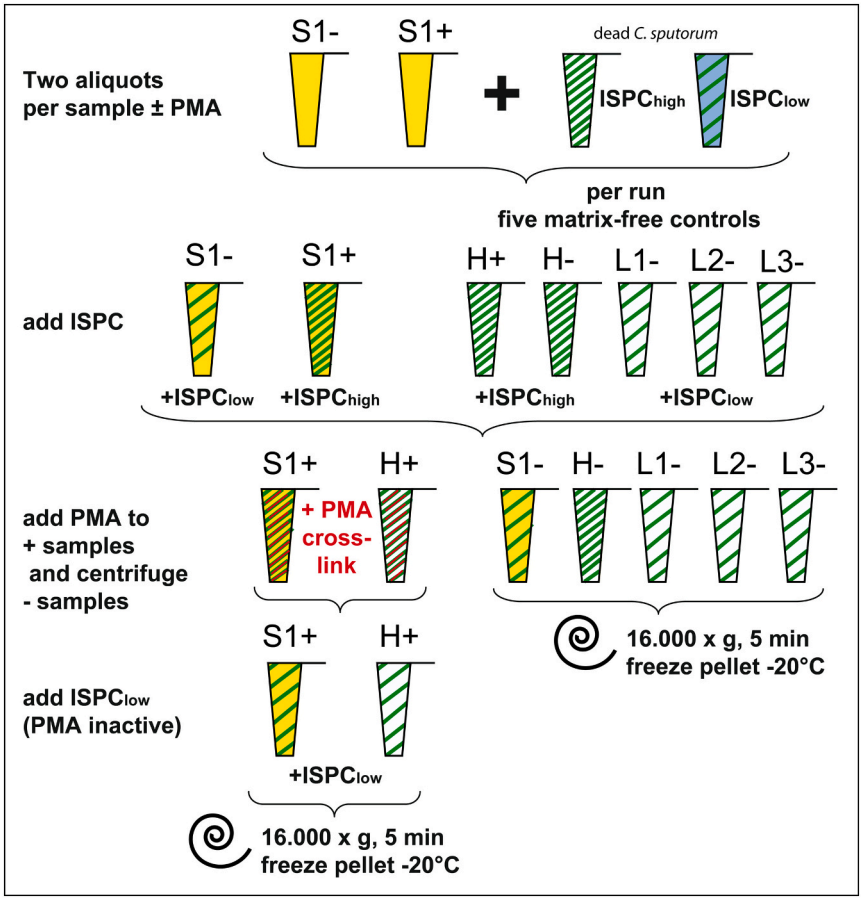

Fig. 1. Overview of the viability qPCR for routine application. Per sample two aliquots were processed, one with PMA (S+) for quantification of live thermophilic *Campylobacter* spp. and the other one without PMA (S-) for the number of total thermophilic *Campylobacter* spp., including dead bacteria. Dead *C. sputorum* cells were used at high concentration $ISPC_{high}$ for the estimation of efficient dead cell signal reduction by PMA treatment and at low concentration $ISPC_{low}$ for estimation of eventual DNA losses during sample processing. Per run, five matrix-free controls in PW were included. Three identical controls harboring $ISPC_{low}$ without PMA (L1-, L2-, L3-) and two additional controls with $ISPC_{high}$, one with and without PMA treatment (H-, H+). S- received $ISPC_{low}$ and was directly centrifuged together with the controls not receiving PMA (L1-, L2-, L3- H-) and the cell pellets were stored at −20 °C before DNA extraction. S+ and H+ received $ISPC_{high}$ and PMA, were incubated at 30 °C for 15 min in the dark before crosslinking of PMA to DNA located in dead cells. In order to be able to assess also DNA losses in the same tube, $ISPC_{low}$ was added after crosslinking and lack of PMA reactivity. $ISPC_{low}$ in the samples is expected to be quantitatively recovered as observed for the matrix-free $ISPC_{low}$ controls (L1-, L2-, L3-). Otherwise, the target signal of thermophilic *Campylobacter* spp. was adjusted to this reduced $ISPC_{low}$ signal in the samples.

2.4), DNA was analysed by triplex qPCR. In parallel, CFU was determined according to ISO 10272-2:2017 using the 1 ml of residual sample.

For determination of the accuracy, eight samples based on a chicken skin with a low organic matter content of 6 mg/ml were tested by spiking different levels of live *C. jejuni* (low, medium, high CFU) and different ratios of live/dead *C. jejuni* (Suppl. Table 2). For each level of contamination, accuracy runs were carried out in five replicates. Each replicate was analysed with and without PMA dye with the alternative method. For all samples at least one of the replicates was also processed with the reference method. For three samples all five replicates were analysed with the reference method.

For the determination of the LOQ, four levels of contamination close to the theoretical limit of four chromosomal copies (theoretically expected number of 7.5, 15, 30 and 45 copies in the PCR reaction) were analysed in three replicates on three experimental days in low (6 mg/ml) and medium (54 mg/ml) organic matter matrix (Suppl. Table 3). Note that three target copies per chromosomal copy are present in *Campylobacter* spp., i.e. three copies of the 16S rRNA gene per chromosome. In order to account for slight variations in the contamination levels on each experimental day, the practically expected numbers of copies were calculated based on the CFU results per day obtained from a 1:4000 dilution of the respective live *C. jejuni* cryovial, corresponding to a mean of 83 colonies (range from 68 to 93 colonies) (Table 1). This number corresponded to the lowest spiking level per ml sample, from which 1:10th was measured in the qPCR reaction for LOQ determination (≙8.3 copies).

2.7. Organization of an international ring trial

In order to fulfil the second part of a method validation study according to ISO 16140-2:2016(E), an international ring trial was conducted with 12 participants. Participants registered using an Excel form and information was gathered on the available real-time PCR instruments with possible probe-dye combinations, the PMA crosslink device and whether microbiological *Campylobacter* enumeration was performed as a routine. Parcels were shipped on 23rd September 2019 and results were obtained until 11th October 2019. The parcels were shipped on dry ice and the participants were asked to confirm proper receipt. In addition to the reference material (live and dead *C. jejuni*, meat rinses, DNA standards, ISPC) also mCCDA powder and CCDA selective supplement (mixture of Merck & Co. and Oxoid powder), PW-blue, PMA, a GeneJet Genomic DNA extraction kit and mastermixes appropriate for two different probe dye combinations (either FAM-Cy5-HEX or FAM-JOE-TAMRA) were provided for performance of the triplex and the two duplex qPCRs. A standard operation procedure (SOP) was provided for the participants. The parcels contained blind-coded samples. In brief, chicken skin rinses of 3 ml (8 mg/ml of organic matter) were requested to be spiked with three different cell cultures: live *C. jejuni* (CS-1), dead *C. jejuni* (CS-2) and a blank vial (CS-3). After thawing the cell suspensions at RT for 30 min and recovery for 60 min on ice, they were used for blind-coded spiking (Suppl. Table 4). Thereafter, the samples were kept at room temperature, if not stated otherwise in the SOP.

After plating the samples on mCCDA for CFU determination, the alternative method was performed as described in Section 2.3. If a halogen lamp of at least 500 W was used for crosslinking, the participants were asked to place the samples in a water bath and locate the lamp in a distance of about 20 cm from the samples in order to prevent heating of the samples (Suppl. Information 1). During the 15 min of photo-activation, the water temperature had to be checked to not exceed 30 °C. Thereafter, the samples were either stored at −20 °C or directly processed as described in Sections 2.4. to 2.6. For the qPCR reactions, the PerfeCTa MultiPlex qPCR ToughMix (Quantabio, Beverly, MA, USA) was used. In the triplex qPCR assay, either the dye combination FAM-Cy5-HEX or the dye combination FAM-JOE-TAMRA was employed for the detection of *C. jejuni*, the ISPC and the internal amplification control (IAC; IPC-ntb2), respectively. For the first of the two duplex qPCR reactions, either FAM-HEX or FAM-TAMRA were used (*C. jejuni* and IAC), and for the second reaction Cy5-HEX or JOE-TAMRA (ISPC and IAC).

2.8. Data analysis

The participants delivered number of CFU per plate and dilution and calculated Ct values as well as raw data. Data of the validation study, including the interlaboratory ring trial data were pre-analysed by BfR and LGL before statistical analysis was performed by QuoData Quality & Statistics, Dresden, Germany. Calculations were conducted according to the principles of ISO 16140-2:2016(E) Sections 6.1.2.3, 6.1.3.3, 6.1.4.4 and 6.2.3. The two variants of the alternative method (duplex and triplex qPCR) were investigated independently.

As only one replicate was measured for sample RT-4 (Suppl. Table 4), repeatability standard deviations could not be analysed for this sample. No false-positive results (blank sample M5) were observed in the data after quality processing (see Section 3.6.1). For each level of contamination i (i.e. for each sample), the means across all laboratories and replicates were calculated for the reference method as well as for both variants of the alternative method, resulting in three sample-specific reference values Xi and six sample-specific average values of the alternative method $\bar{y}_i$ (three for each qPCR variant). Then the sample-specific biases Bi between the alternative method and the reference method were calculated using the respective mean values of the reference and the alternative method. In addition, the repeatability standard deviation $s_{r,i}$ between-lab standard deviation $s_{L,i}$ and the reproducibility standard deviation $s_{R,i}$ were determined for all method/sample combinations. Based on these statistical values and taking the number of replicates $n = 2$ and the number of laboratories $p = 9$ (number of laboratories regarding each variant of the alternative method) into account, the upper and lower β-expectation tolerance interval limits (β-ETI[2]) were calculated for each sample. Furthermore, analyses were done on robust statistics (Q/Hampel method as described in ISO 13528:2015, as, in

Table 1
Mean of detected copies of live *C. jejuni* counts with low and medium organic load in comparison to the theoretically and practically expected number of copies.

Theoretically expected number of copies (based on calculation)	Practically expected number of copies (based on CFU)[a]	Mean number (empirical) of detected copies for LOQ (triplex qPCR)[b]		Recovery (%)	
		Low organic material concentration	Medium organic material concentration	Low organic material concentration	Medium organic material concentration
7.5	8.3	8.9	5.7	108	68
15.0	16.5	16.2	8.8	98	54
30.0	33.0	36.3	19.8	110	60
45.0	49.6	47.5	35.5	96	72
Mean recovery				103	63

Note: $ISPC_{low}$ was omitted from this experiment, thus, DNA losses were not accounted for.
[a] For each experimental day, the lowest concentration was determined by CFU determination, from which the higher concentrations were calculated.
[b] Based on three experimental days (9 replicates), except of value 8.9 (only 6 replicates).

particular, the measured values for the direct CFU determination and the reference method were quite inhomogeneous.

3. Results

3.1. Concept for challenging CFU by live/dead differentiating qPCR

The principle of the live/dead differentiating qPCR method for thermophilic *Campylobacter* spp. is to add an internal sample process control (ISPC; a distinct number of dead *C. sputorum* cells) to the samples, which enables (i) monitoring the effective reduction of dead cell signal (achieved by the photo-activated DNA intercalating dye PMA, which interferes with amplification of dead cells' DNA during PCR) and (ii) compensation for eventual DNA losses during processing (Pacholewicz et al., 2019). In this way, intact and putatively infectious units (IPIU), comprising CFU and VBNC, are detected for improved food safety.

Biological food safety is usually assessed by the analysis of CFU. We wanted to establish the novel viability real-time PCR method suitable for practical use in quantification of live thermophilic *Campylobacter* spp. on food matrices. Therefore, we performed a validation study according to ISO 16140-2:2016(E). This ISO norm demands a direct comparison of the novel method against the current gold standard, which is the microbiological enumeration method ISO 10272-2:2017.

3.2. Stabilization, quality control and application of ISPC

As a prerequisite for practical use of the ISPC-controlled v-qPCR, we established a protocol for the long-term storage of the ISPC control. The ISPC corresponds to a distinct number of dead *C. sputorum* cells. *C. sputorum* cells did not grow using gas-generation systems but only displayed reproducible growth in either a microaerobic incubator or in vacuum jars filled with an appropriate gas mixture (5% O_2, 10% CO_2, rest N_2). After growth, *C. sputorum* bacteria were killed by oxidative stress and transferred to a cryomedium, containing trehalose and glycerol as well as FBS for optimal long-term protection of DNA within the dead bacterial cell compartment. A lyophilisation protocol was performed for stabilization of the ISPC. Homogeneity check was performed on 10 vials in duplicate and a mean $\log_{10}$ copies of *C. sputorum* genomic equivalents of 5.98 ± 0.07 was obtained by duplex qPCR in the absence of PMA. The ISPC was homogenous according to ISO 13528:2015.

We further checked, whether the concentration-dependent kinetics of PMA signal reduction, as an indicator for DNA accessibility to the intercalating dye, was changed during time and storage conditions (Fig. 2). In the absence of PMA, the starting concentration of ~6 $\log_{10}$ per ml (of 1000-fold diluted ISPC) was confirmed. As a control, extracted free genomic DNA of *C. sputorum* was included. After 6, 9, 12 and 18 months of storage at 4 °C, 25 °C or 37 °C, ISPC vials were reconstituted in PW and 10^6 cells were titrated with PMA for quality and stability assessment. As expected for the control of free DNA, the PCR signal was strongly reduced at PMA concentrations up to 1 μM. The subsequent rise in PCR signal of free DNA at higher PMA concentrations might be due to steric hindrance and inefficient PMA crosslinking due to PMA-oversaturated DNA. The signal reduction kinetics of the ISPC stored at 37 °C was similar to free DNA at concentrations up to 1 μM. This suggests that the main fraction of DNA of this ISPC was similarly accessible to PMA as observed for free DNA. Storage at 4 °C showed completely different kinetics of PMA concentration-dependent reduction of the PCR signal, reaching a minimal plateau at concentrations above 5 μM PMA. Since it was verified that the H_2O_2-inactivated *C. sputorum* cells lacked CFU within $\geq 10^8$ cells (no survivor within 10^8 plated bacteria) even before lyophilization, the kinetics indicate that DNA in dead cells still displayed some residual signal at PMA concentrations ≥5 μM and that the ISPC stored at 4 °C was stable for at least 18 months. As expected, the ISPC stored at 25 °C showed an intermediate signal reduction curve that was between the ISPC stored at 4 °C and the one stored at 37 °C.

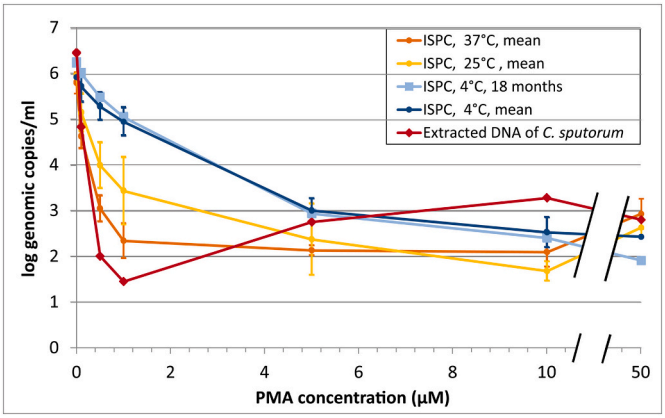

Fig. 2. Kinetics of DNA rest signal upon titration of PMA reflects ISPC stability at 4 °C for at least 18 months. ISPC lyophilisates were stored at different temperature conditions. After 6, 9, 12 and 18 months the ISPC was reconstituted in PW and 10^6 cell counts were analysed in PBS in the presence of different concentrations of PMA. Log_{10} genomic copies per ml were obtained by real-time PCR analysis. As control for free/extracellular DNA, extracted genomic DNA of *C. sputorum* was challenged by PMA at similar concentrations (one replicate). Mean values and standard deviations are depicted from at least three experiments, with the colour code for storage at 37 °C in orange, 25 °C in yellow and 4 °C in blue. Light blue, timepoint 18 months at 4 °C is highlighted. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

Furthermore, the standard deviation was smallest for the 4 °C condition, supporting maximal stability of ISPC under this condition. Thus, we provide a quality and stability check of the ISPC and conclude that storage of the lyophilisate is most appropriate at refrigerating conditions of 4 °C.

Previously, the ISPC was added at the same high concentration to each of the two sample aliquots. The aliquot receiving PMA was used to monitor appropriate dead cell signal reductions and IPIU counts of thermophilic *Campylobacter* spp., while the other aliquot (sample without PMA) served for estimation of putative DNA losses (Pacholewicz et al., 2019). However, during method optimization, we occasionally observed that due to intra-sample variations, normalization of DNA loss estimated from the second aliquot did not necessarily reflect DNA loss observed in the first aliquot. Hence, we optimized the method in order to obtain both read-outs, the final IPIU and DNA loss in the same aliquot, while estimating a sufficient dead cell signal reduction by comparing the aliquot with and without PMA. For this purpose, the aliquot receiving PMA first obtained a high concentration of ISPC ($\text{ISPC}_{\text{high}}$), expected to be quenched for at least 2.5–3 $\log_{10}$ upon PMA treatment. Subsequent to crosslinking and loss of PMA reactivity, a low concentration of ISPC (ISPC_{low}) was added. This concentration was chosen just above the expected residual $\text{ISPC}_{\text{high}}$ signal after PMA treatment, in order to be able to normalize to ISPC_{low} signal in matrix-free controls (mean of L1-, L2-, L3-) (Fig. 2). For visual differentiation and guidance for exact pipetting, the ISPC_{low} was prepared from $\text{ISPC}_{\text{high}}$ by dilution into PW-blue. We proved that the blue colour (bromophenol blue) did not exert any unwanted effect on the qPCR method by quantifying comparable numbers of *C. sputorum* genome copies in stained and non-stained ISPC_{low}.

3.3. Design of the triplex qPCR for practice application

Moreover, the former two duplex PCRs, either targeting ISPC and internal amplification control (IAC) or thermophilic *Campylobacter* spp. and IAC (Pacholewicz et al., 2019), were integrated into a single triplex qPCR. The triplex qPCR detects thermophilic *Campylobacter*, the ISPC and the IAC in one reaction. Since a signal of ISPC_{low} of around 1000 copies per reaction is expected in the samples, the *C. jejuni* DNA standard curve contained serial dilutions of *C. jejuni* genomic equivalents in the

presence of 1000 genomic copies of ISPC (*C. sputorum* genomic equivalents) named Cjs1-5.

The performance characteristics of the triplex qPCR were similar to the two duplex qPCRs (Table 2 and Pacholewicz et al., 2019), with a mean PCR efficiency ≥95% and a mean correlation coefficient ≥ 0.99 calculated from standard curves performed in the two different laboratories (Table 2, $n_{Cjs} = 33$, $n_{Sp} = 28$). In addition, the ISPC background of 1000 copies in the Cjs standard was reliably detected with a mean recovery of 93%. For the application of the validated v-qPCR, we defined the following performance criteria for reliable quantitative detection of *Campylobacter* spp.: (i) standard slopes should range between −3.1 to −3.6 (corresponding to 90–110% efficiency), (ii) correlation coefficients of both standards should correspond to ≥0.98 and (iii) the ISPC background signal in the Cjs1-5 standard series should account for 3 ± 0.3 $\log_{10}$ genomic copies of *C. sputorum*. We validated the method using two master mixes, either the single component master mix using the Platinum™ Taq Polymerase at 2 IU per reaction (Thermo Fisher Scientific Inc.) or the PerfeCTa MultiPlex qPCR ToughMix (Quantabio, Beverly, MA, USA). The performance was checked for each PCR run (e.g., by using the provided data analysis file in Suppl. Information 3). Thus, also other master mixes may be used with similar performance characteristics, which should be confirmed using the Cjs1-5 standard series. In certain samples with a high load of thermophilic *Campylobacter*, a competition of the *Campylobacter* signal on the ISPC signal is expected (see also sample No. RT-4 of the interlaboratory study), and the two duplex qPCRs are preferable. However, we expect that for most native samples with viable *Campylobacter* counts around or below the process hygiene criterion, the triplex qPCR is the method of choice.

3.4. General considerations for validating the alternative culture-independent v-qPCR

The alternative v-qPCR method was validated according to ISO 16140-2:2016(E). This ISO norm mandates to validate the alternative method against the reference method (ISO 10272-2:2017). The original idea of developing a culture-independent method for quantification of live *Campylobacter* spp. was to overcome weaknesses of the reference method, e. g. labour- and time-intensive performance of the microbiological enumeration method but also the lack of culturability of stressed but still viable *Campylobacter*, not capable to form a colony on an agar plate. Thus, in order to validate an improved alternative method against a reference method with known drawbacks, the crucial point is to start from a highly active quantitative *C. jejuni* standard, from which reproducible CFU values were obtained. A prerequisite for a successful validation is the distribution of homogenous and stable CFU material to the participants (NRL for Campylobacter, 2021). The cryovials used in our study passed homogeneity tests and were stable for at least 18 months. CFU analysis revealed slight variations of CFU recovery on different agar media. On Oxoid mCCDA a concentration of 5.0 ± 0.41 $\log_{10}$ CFU/ml of *C. jejuni* was measured ($n = 9$). On Columbia blood agar we obtained 5.33 ± 0.25 $\log_{10}$ CFU/ml ($n = 10$) and on Merck mCCDA 5.51 ± 0.08 $\log_{10}$ CFU/ml (n = 9) were obtained. Since we observed that the Oxoid mCCDA had slightly improved inhibition capacity against background flora present in chicken rinses whereas Merck agar provided improved CFU recovery of *Campylobacter* spp., we provided a mixture of dry powder of mCCDA from Oxoid and Merck to each participant of the interlaboratory ring trial. The data further implied that CFU determination was strongly dependent on optimal growth conditions. Furthermore, dead *C. jejuni* cells were produced with genomic equivalents of 5.9 ± 0.12 $\log_{10}$ per ml ($n = 12$). In addition, meat rinse matrices with different loads of organic matter, mimicking native samples in routine laboratories, were prepared from *Campylobacter*-free material.

Table 2
Performance characteristics of the triplex qPCR.

C. jejuni standard (mean values)	
Slope	−3.4
Cor. coef. (R^2)	0.993
Efficiency in %	95.41
n Standard curves	33
ISPC standard (mean values)	
Slope	−3.4
Cor. coef. (R^2)	0.991
Efficiency in %	99.4
n Standard curves	28
ISPC signal stability in Cjs standard	
Theoretical number of copies	1000
$\log_{10}$ (theoretical no. of ISPC copies)	3
No. of standard curves	13
No. of values	113
Mean no. of ISPC copies ($\log_{10}$)	2.97
Standard deviation (SD) ($\log_{10}$)	0.13
Mean no. of ISPC copies + 2 SD ($\log_{10}$)	3.22
Mean no. of ISPC copies − 2 SD ($\log_{10}$)	2.71
Mean recovery of ISPC	93%

For quantitative analysis, quality criteria should be as follows: efficiency between 90 and 110%; correlation coefficient of ≥0.98 and ISPC recovery at 3 ± 0.3 $\log_{10}$.

3.5. Method comparison study

ISO 16140-2:2016(E) demands the validation to be conducted in two parts, a method comparison study, which was performed in two laboratories and an interlaboratory ring trial. The method comparison study was separated into four experimental steps. An inclusivity and exclusivity study had been performed, determining the qPCR targets specificity and sensitivity (Pacholewicz et al., 2019). A relative trueness study was conducted in order to determine the robustness of the method against matrix variations (Section 3.5.1). Furthermore, the limit of quantification was determined (Section 3.5.2) and finally, the accuracy of the method was specified (Section 3.5.3).

3.5.1. Relative trueness study – robustness against matrix variations

Within the relative trueness study, the v-qPCR method was challenged by the use of different types of raw meat matrices, relevant for quantification of thermophilic *Campylobacter* spp. We chose (i) raw chicken meat, (ii) raw chicken skin and (iii) raw minced pork/beef meat. Different matrix rinses were prepared by the addition of various volumes of PBS to meat samples and manual rinsing as well as stomacher homogenization, resulting in 15 distinct rinse samples with different wet weights of organic matter ("M-No." sample names, Suppl. Table 1). The $\log_{10}$ IPIU/ml values obtained with the alternative triplex qPCR method were slightly higher than the values obtained with the colony-count method (Fig. 3) since all except four data points lie above the line of identity. On average, the mean number of IPIU determined by the alternative method exceeded the CFU values by 0.22 $\log_{10}$ counts/ml. Based on this mean difference, the standard deviation of the differences of 0.26 $\log_{10}$/ml and the number of samples ($n = 21$), the limits of agreement were calculated according to ISO 16140-2:2016(E), Section 6.1.2.3 with the lower limit of −0.33 $\log_{10}$ counts/ml and the upper limit of 0.78 $\log_{10}$ counts/ml. Since it was expected that not more than one in 20 data values will lie outside the limits, one outlier in 21 data values (Fig. 3, R2-7) was in agreement with the expectations.

3.5.2. Accuracy

Based on the median values of the reference method *Xi* and the alternative method *Yi*, the deviation of the alternative method from the reference method (absolute bias *Bi*) was calculated for all samples. In addition, the standard deviation of the alternative method s_{alt} was determined across all samples ($s_{alt} = 0.086$). The upper and lower

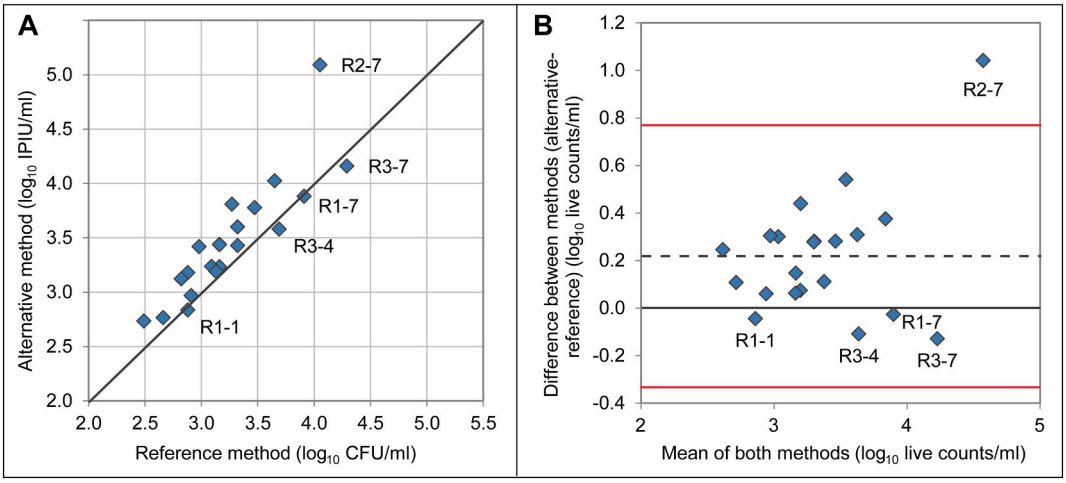

Fig. 3. Results of the trueness study on raw meat samples show that the alternative v-qPCR method lies within the limits of agreement. A, scatter plot, illustrating the correlation between the alternative triplex qPCR method versus the reference enumeration method ISO 10272-2:2017 on various meat rinses; B, Bland-Altman difference plot, showing one outlier (R2-7) in 21 data values, which is in agreement with the expectations set in ISO 16140-2:2016(E). Solid black line, line of identity; dashed black line, mean difference (+0.22 $\log_{10}$ live counts), red lines indicate lower (−0.33 $\log_{10}$ live counts) and upper limit of agreement (0.78 $\log_{10}$ live counts). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

β-expectation tolerance interval (β-ETI) limits were calculated for each sample. A summary of all relevant statistical results is provided in Table 3. It can be observed that there is no obvious systematic bias. The β-expectation tolerance interval lies entirely within the acceptability limits of ±0.50 $\log_{10}$ live counts per ml, and thus, in accordance with ISO 16140-2:2016(E), the alternative method is accepted as being equivalent to the reference method regarding raw meat rinses.

3.5.3. *Determination of the limit of quantification (LOQ)*

For LOQ determination, only the samples treated with PMA were considered, as only those results were comparable to the CFU values. Two chicken rinse matrices with different loads of organic material (low and medium organic load) were spiked with live *C. jejuni*. The theoretically expected number of copies (based on calculations) and the practically expected number of copies (based on CFU determination) were similar (Table 1). For the low concentration of organic material, also the number of detected copies in the triplex qPCR was close to the expected number of copies (theoretically and practically expected number, Table 1). Taking the practically expected number of copies as a reference and applying a linear model, the recovery is not significantly different from 100% (Fig. 4A, orange line). However, for the medium concentration of organic material, it was only possible to recover a portion of the expected number of copies: Applying a mixed linear model, a recovery of 63% was estimated, which is significantly lower than 100% at a significance level of 1% (Fig. 4A, blue line). The reduced recovery at higher concentrations of organic material could be due to some inhibitory effects by the organic load of the matrix, reducing the efficiency of the DNA extraction and/or the PCR amplification process and making quantification more difficult. For the LOQ determination, only $ISPC_{high}$ was applied for monitoring PMA-dependent dead cell signal reduction. Thus, eventual DNA losses in the sample aliquot receiving PMA, in particular expected for medium organic loads, was not observed due to lack of $ISPC_{low}$ at that stage of method development. Thus, the determined LOQ displays the upper limit of possible detection and the final method is supposed to even have an increased performance by including also $ISPC_{low}$.

For colony-count techniques, a theoretical repeatability standard deviation is in general expected to be characterized by the Poisson

Table 3
Results for the accuracy profile study regarding raw meat rinses (chicken skin).

Sample	Central value ref. method X_i [$\log_{10}$ live counts/ml]	Central value alt. method Y_i [$\log_{10}$ live counts/ml]	Absolute bias B_i [$\log_{10}$ live counts/ml]	Upper β-ETI[b] U_i [$\log_{10}$ live counts/ml]	Lower β-ETI[b] L_i [$\log_{10}$ live counts/ml]	Upper AL $+AL$ [$\log_{10}$ live counts/ml]	Lower AL $-AL$ [$\log_{10}$ live counts/ml]
A-1	2.20	2.33	0.13	0.25	0.01	+0.50	−0.50
A-2	2.77	2.69	−0.08	0.05	−0.21		
A-7	3.07	3.13	0.06	0.18	−0.06		
A-3	3.31	3.31	0.00	0.12	−0.12		
A-8	3.90	4.01	0.11	0.23	−0.01		
A-4	3.92	3.62	−0.30	−0.18	−0.43		
A-6	4.37	4.48	0.11	0.24	−0.01		
A-5	4.50	4.18	−0.32	−0.20	−0.44		
Standard deviation of the reference method s_{ref}				0.092 $\log_{10}$ live counts/ml[a]			
Standard deviation of the alternative method s_{alt}				0.086 $\log_{10}$ live counts/ml			

Data sorted by the central value determined by the reference method.

[a] Calculation of s_{ref} is based on only three instead of eight samples (A-1, A-7 and A-8), thus s_{ref} is neither comparable to s_{alt} nor representative of the food type and category.

[b] β-ETIs are the intervals where it is expected that a proportion β (here: 80%) of future measurements will fall inside (see ISO 16140-2:2016(E), Section 6.1.3.3 and annex G.4).

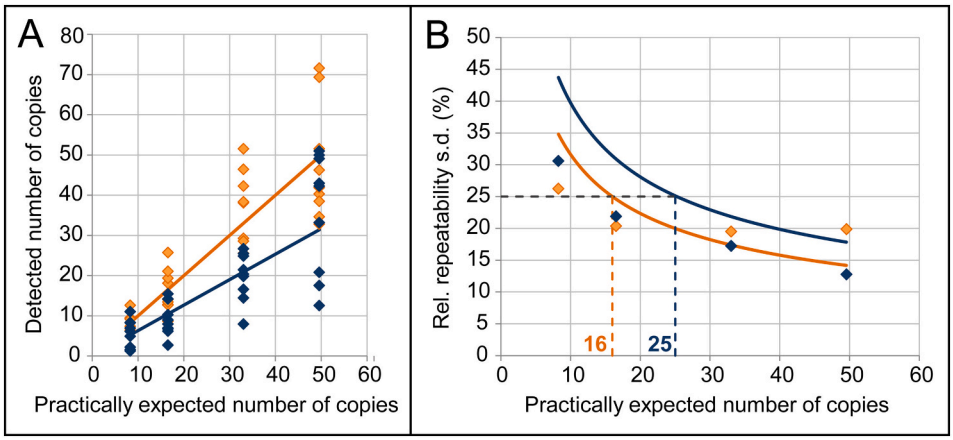

Fig. 4. The LOQ was determined to be 16 to 25 copies per test portion depending on the organic material concentration (low or medium). A, For the low organic material concentration, the detected number of copies was similar to the practically expected number (recovery not significantly different from 100%); for the medium organic material concentration, a recovery of 63% was estimated. B, Relative repeatability standard deviation in dependence of the practically expected number of copies as well as the theoretical relative repeatability standard deviation derived from the Poisson distribution for both organic material concentrations. Orange, low organic material concentration; blue, medium organic material concentration; diamonds, empirical values; lines, theoretical values. In the case of chicken rinse with medium organic load, the theoretical repeatability standard deviation is recovery corrected. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

distribution. To test whether this also applies to the triplex qPCR, the empirical repeatability standard deviation (mean standard deviation within one experimental day for each sample) was extracted considering the 3 × 3 experimental design (three replicates on three experimental days). In Suppl. Fig. 1, the empirical repeatability standard deviations were compared to the repeatability standard deviations expected from the Poisson distribution. At first glance, the empirical repeatability standard deviations seemed to deviate from the theoretical repeatability standard deviations. However, considering the 95% confidence intervals (shown as whiskers in Suppl. Fig. 1) obtained by bootstrapping, no significant differences were detected, as the line of identity was included in the confidence intervals for all samples. The empirical relative repeatability standard deviations with regard to the practically expected numbers are shown in Fig. 4. This figure also shows the theoretical relative repeatability standard deviations characterized by the Poisson distribution. It also takes into account the recovery, from which an LOQ can be derived for both organic material concentrations. For the low organic material concentration, a correction of the repeatability standard deviation was not necessary. In contrast, for the medium organic material concentration, a recovery of 63% was observed, resulting in a shift of the curve with regard to the repeatability standard deviation derived from the Poisson distribution. Assuming that a quantitative determination was only ensured if the relative repeatability standard deviation did not exceed 25%, this resulted in a limit of quantification of 16 genomic copies per test portion for the low organic material concentration, and 25 genomic copies per test portion for the medium organic material concentration (Fig. 4B).

3.6. International ring trial

In order to fulfil the validation criteria demanded in ISO 16140-2:2016(E), an interlaboratory ring trial was organized for the quantification of live thermophilic *Campylobacter* in chicken rinse. For this purpose twelve participating laboratories were contacted, which had either experience in routine microbiological quantification of *Campylobacter* spp. or in routine quantitative real-time PCR analysis or ideally in both techniques. The participating laboratories were from France, Germany, Iceland, Poland, Slovenia, The Netherlands, United Kingdom, and USA. A standard operation procedure was provided in order to optimally guide the experimental operators to examine blind-coded samples using the reference method described in ISO 10272-2:2017 as well as the two variants of the alternative method (the duplex and the triplex qPCR). In addition, a direct CFU determination of the cell suspensions used for spiking was performed as well.

3.6.1. Data basis

All participants performed the reference method as well as the triplex qPCR and the duplex qPCR, and all laboratories, except for laboratories 004, 008 and 012 also performed the direct counting of the spiking suspensions. Laboratory 006 submitted data based on two sets of fluorophores for both the duplex and triplex qPCR: The data set 006a was based on the combination of the probes HEX, FAM and Cy5, and the data set 006b on the combination of the probes FAM, JOE and TAMRA. However, laboratory 006 had reported in advance that the combination of the three probes used for 006b currently posed problems in their PCR assays, and the required qPCR quality criteria could not be fulfilled for the triplex qPCR, leading to exclusion of this data set. To avoid duplicate data sets produced by the same laboratory, we decided to exclude the duplex qPCR data set 006b from further analyses as well.

In addition, we excluded datasets due to methodological shortfalls (in accordance with ISO 16140-2:2016(E)). The methods used by the participating laboratories are listed in Suppl. Table 5. The laboratory/method combinations excluded from the analysis are marked in light red. PCR datasets of laboratory 001 (duplex and triplex) and 004 (only triplex) were omitted due to failed DNA standard curve quality criteria (efficiency between 90 and 110% and a correlation coefficient of $\leq$0.98). Duplex qPCR data were also omitted from laboratory 003 because of a false positive result of the negative control, indicating contamination of this PCR, while the triplex qPCR was unaffected. Data from laboratory 005 (reference method as well as both variants of the alternative method) were discarded, since the data were not obtained within the requested time window of the interlaboratory study and since both qPCR variants did not meet the required performance criteria. Direct plating from the spiking solutions of laboratory 006 did not yield any colony, and was omitted as well.

3.6.2. Performance parameters obtained from the interlaboratory ring trial

The participants of the interlaboratory ring trial examined blind-coded samples. These included three identical samples with different contamination levels of live and dead *C. jejuni* in duplicate (Suppl. Table 4), as well as one negative control and one sample with a high load of dead *C. jejuni*, expected to cause pitfalls in the triplex qPCR. In Fig. 5, the results of the participants for the three replicate samples and the two additional single samples (negative control (blank) and sample RT-4 with a high background load of dead *C. jejuni*) are illustrated. The qPCR data of live *Campylobacter* spp. (Fig. 5, orange boxes for triplex qPCR and yellow boxes for duplex qPCR) are depicted side-by-side to the reference method of CFU determination from the respective samples (Fig. 5, blue boxes) and the direct CFU determination from the spiking

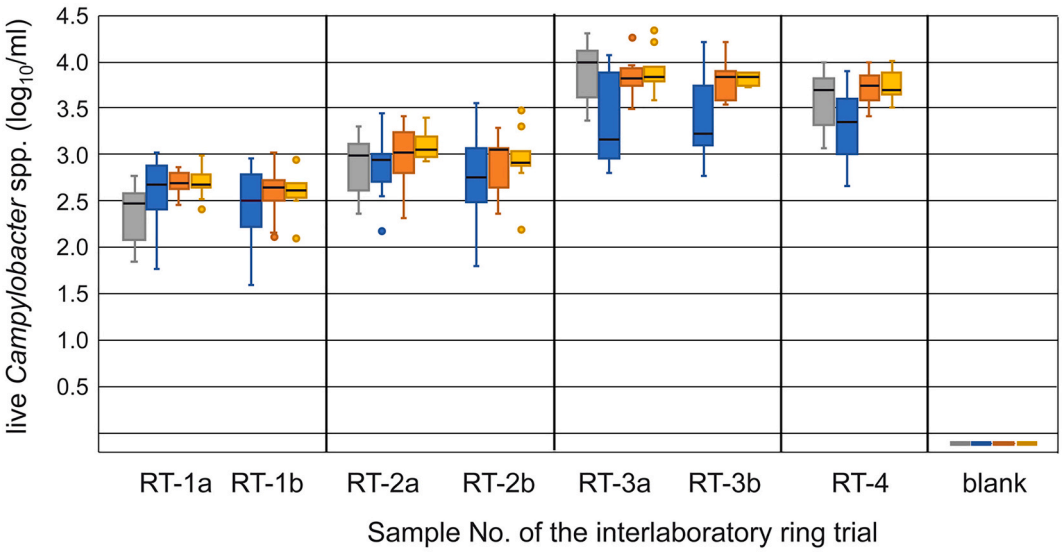

Fig. 5. Boxplot of live *Campylobacter* spp. determined in the interlaboratory ring trial. Sample numbers are according to Suppl. Table 4. Samples "a" are identical replicates of the respective sample "b". Grey, direct CFU determination results from the spiking solution; blue, CFU determination from samples; orange, IPIU determined by triplex qPCR; yellow, IPIU determined by duplex qPCR. The boxplot length corresponds to the interquartile range (IQR) of data (50% of the data), the horizontal bar indicates the median value; dots, outliers (>1.5 × IQR below the first quartile or above the third quartile); whiskers represent 1.5 × IQR or the maximum/minimum value of the dataset. The fraction of dead *C. jejuni* in RT-4 exceeded the limit of the triplex qPCR and inhibited the ISPC signal in PMA-untreated sample. The depicted values of live *C. jejuni* are, therefore, the maximal number of live *C. jejuni* in RT-4 but correspond well with the duplex qPCR data and the direct CFU determination from the spiking solution. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

solution (Fig. 5, grey boxes). The variance of the data from the CFU methods was considerably higher than for the qPCR methods. CFU appeared lower with higher amounts of spiking levels in the sample matrix. In contrast, v-qPCR data keep consistency with the direct CFU measurements of the spiking solution, independent of the spiking level.

In RT-4, the fraction of dead *C. jejuni* exceeded the limit of the triplex qPCR (5.7 $\log_{10}$ per ml; Suppl. Table 4) and inhibited the ISPC signal in the PMA-untreated sample. Hence, putative DNA losses in this PMA-untreated sample could not be monitored and the true value of dead cells measured by the triplex qPCR equalled or was higher than the value obtained from the PMA-untreated sample. As a consequence, it cannot be excluded that the value of live *C. jejuni* was partly influenced by residual signal from dead cells, as the absolute number of dead cells in the PMA-untreated sample was unknown. In this case, we recommend the performance of the two duplex qPCRs, in which a competition of the target amplification for thermophilic *Campylobacter* with the amplification of the ISPC is excluded.

For each sample, the means across all laboratories and replicates were calculated for each method, in order to be able to measure the absolute bias between reference and alternative methods and the upper and lower β-expectation tolerance interval limits (β-ETI) (Table 3, Fig. 6). In addition, for the three identical duplicate samples (RT-1, RT-2 and RT-3), the repeatability standard deviation, between-lab standard deviation and the reproducibility standard deviation were determined for all method/sample combinations according to ISO 16140-2:2016(E). As expected from the validation data of the reference method (Jacobs-Reitsma et al., 2019), the reference method suffered from strong variance. Hence, the accuracy values were also calculated for all four samples (including RT-4) based on the robust mean (Q/Hampel method described in ISO 13528:2015) and compared with direct CFU determination from the spiking solution (Suppl. Table 6).

The reproducibility standard deviations were larger for the reference method and the direct CFU determination than for the alternative qPCR methods (Table 4). For the reference method, the reproducibility standard deviation was between 0.39 and 0.48 $\log_{10}$ CFU/ml. In contrast, the reproducibility standard deviation was between 0.23 and 0.29 $\log_{10}$ IPIU/ml for the two qPCR methods – except for RT-2, triplex qPCR, with

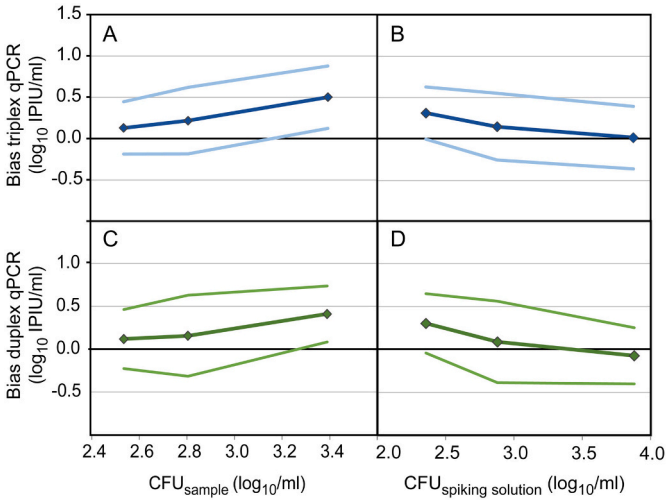

Fig. 6. Accuracy profile from the interlaboratory study. Accuracy was assessed via calculating the bias of the two qPCR methods in comparison to the reference method (CFU determination from the samples) (A, C) and on direct CFU determination from the spiking solution (B, D). A and B (in blue), triplex qPCR; C and D (in green), duplex qPCR; solid black line, line of zero bias; dark blue or green lines, absolute bias; light blue or green lines, upper and lower β-ETI. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

0.33 $\log_{10}$ IPIU/ml according to ISO 16140-2:2016(E). However, the larger reproducibility standard deviation was mainly caused by one of the two measurement values in the data set of one laboratory. But even though trends seemed obvious, the differences between the methods were not statistically significant.

The repeatability standard deviation increased with increasing contamination from 0.14 to 0.22 $\log_{10}$ CFU/ml for the reference method. The opposite was true for both qPCR methods. Here, the repeatability standard deviation showed a decreasing trend with increasing spiking

Table 4
Performance parameters obtained from the interlaboratory ring trial.

Sample no.	Number of laboratories	Live *C. jejuni* (theoretical) [log_{10}/ml]	Mean live *C. jejuni* (measured) [log_{10}/ml]	Repeatability s.d. [log_{10}/ml]	Between-lab s.d. [log_{10}/ml]	Reproducibility s.d. [log_{10}/ml]
Colony-count technique ISO 10272-2:2017 (reference method)						
RT-1	11	2.48	2.56	0.14	0.37	0.39
RT-2	11	3.00	2.82	0.17	0.39	0.43
RT-3	11	4.00	3.39	0.22	0.43	0.48
Pooled average reproducibility standard deviation $s_{R,\ ref}$						0.43
Duplex qPCR (alternative method variant 1)						
RT-1	9	2.48	2.68	0.17	0.15	0.23
RT-2	9	3.00	3.04	0.210	0.20	0.29
RT-3	9	4.00	3.86	0.08	0.25	0.26
RT-4	9	3.70	3.76	–	–	–
Triplex qPCR (alternative method variant 2)						
RT-1	9	2.48	2.66	0.21	0.13	0.25
RT-2	9	3.00	2.97	0.13	0.30	0.33
RT-3	9	4.00	3.79	0.08	0.21	0.23
RT-4	9	3.70	3.71	–	–	–

Reproducibility, between-lab and repeatability standard deviations were calculated according to ISO 16140-2:2016(E).

level, ranging from 0.21 to 0.08 log_{10} IPIU/ml (duplex qPCR) and from 0.21 to 0.08 log_{10} IPIU/ml (triplex qPCR) (Table 4).

Fig. 6A and C show the resulting accuracy profiles based on the reference method from CFU analysis of the samples. The bias was positive for all samples and both variants of the alternative method, with values between 0.12 and maximally 0.5 log_{10}/ml. It increases with higher reference values, i.e. the number of live *Campylobacter* is always larger for both variants of the alternative method than for the reference method. Increasing numbers of live *Campylobacter* in the sample lead to a stronger increase in the values of the alternative method variants than in the values of the reference method, as also observed in Fig. 5. Due to the strong bias and relatively broad β-expectation tolerance interval, the tolerance interval exceeds the pre-set upper acceptability limit of 0.5 log_{10}/ml. However, in this case, ISO 16140-2:2016(E) allows for an alternative calculation of the acceptability limits as a function of the pooled average reproducibility standard deviation of the reference method (Table 4). Applying the new acceptability limits, the β-expectation tolerance intervals of both variants of the alternative method lie completely within the acceptability limits of ±1.43 (Fig. 6A, C). Thus, following ISO 16140-2:2016(E), both variants of the alternative method can be regarded as fit for purpose regarding raw meat rinses.

We further questioned whether the observed positive bias of the alternative methods was a true bias or whether it was caused by an under-estimation of the reference method as already indicated above (Fig. 5). An estimate for the true contamination level is the CFU value originating from direct CFU determination of the spiking solutions of live *C. jejuni*. This value is independent of matrix effects caused by the food type, and all dilution levels are calculated based on a single dilution level, thus these values intrinsically scale with the contamination level. Using these values, the bias becomes smaller and now decreases with increasing contamination (Fig. 6B and D). The highest β-ETI limit would now be at 0.63 instead of 0.88 log_{10} live *Campylobacter*/ml.

4. Discussion

Microbiological risk assessment (MRA) of food is based on the concept that competent authorities, food business operators and their mandated public or private laboratories rely on estimates of numbers of live infectious cells. As input of an MRA, mainly CFU data, i.e. data on growth of bacteria on agar plates under standard laboratory conditions are used. This concept has been challenged by various recent publications, showing that absence of CFU under certain laboratory conditions does not necessarily reflect absence of regrowth capacity and/or infectious potential (Baffone et al., 2006; Cappelier et al., 1999; Chaisowwong et al., 2012; Federighi et al., 1998; Wulsten et al., 2020). However, accredited use of detection and quantification methods is absolutely needed for harmonized and legal compliance. A novel method should not only be validated against the reference method but should also be practicable and cost-effective for routine laboratories. In our study, we accomplished a method optimization for routine application and presented the international validation of an alternative real-time PCR method for quantification of thermophilic *Campylobacter* spp. in meat rinses according to ISO 16140-2:2016(E).

The optimization of the method comprised the integration of the two duplex qPCR assays into one triplex qPCR assay, the idea of a second addition of a low concentrated ISPC in the same sample after PMA treatment for monitoring DNA losses during sample preparation, and the stabilization of the ISPC for potential distribution and commercialization of the ISPC. The triplex qPCR is suitable for most of the samples. However, if the maximal detection limit of 4.7 log_{10} genome equivalents of thermophilic *Campylobacter* spp. per ml is exceeded (e.g. by excess of dead *Campylobacter* as realized in the RT-4 sample), this signal cannot be judged quantitatively, since the ISPC signal is inhibited and cannot be used for putative quantitative normalization of DNA losses in the sample. In this case, two duplex qPCR assays can overcome this limit. We think that low and medium organic loads as defined in our study resemble the organic load in routine rinse samples. However, overload by organic matter in samples will be monitored by the ISPC, which indicates insufficient dead cell signal reduction by PMA treatment as well as loss of DNA by inadequate DNA extraction.

The results of the method comparison study and the international ring trial showed that the alternative v-qPCR method is fit for purpose, which is the quantification of live thermophilic *Campylobacter* in meat rinses. We did not observe any significant difference in the performance of the triplex qPCR and the two duplex qPCRs within the range of DNA standard concentrations (2 log_{10} to 4.7 log_{10} per ml). The LOQ was around 20 genomic equivalents per reaction, i.e. 200 thermophilic *Campylobacter* spp. per ml of meat rinse, dependent on the organic load of the sample. Since the process hygiene criterion was set to 1000 CFU *Campylobacter* spp. per g of chicken skin, an initial rinse suspension of 1:2 or 1:3 (chicken skin:diluent) is recommended as sample material. We provide an Excel sheet for primer-probe, master mix and DNA standard calculations (Suppl. Information 2), a step-by-step standard operation procedure (SOP, Suppl. Information 1) and an Excel sheet for the analysis of the data based on the SOP (Suppl. Information 3). This will enable routine laboratories to apply this method for sample processing.

During validation it became apparent that the reference method of microbiological enumeration of *Campylobacter* spp. (ISO 10272-2:2017) exhibits strong variance, which was also detected during validation of the reference method (Jacobs-Reitsma et al., 2019). Also, on different

agar media, we observed variance in CFU from the same cryovial, ranging over 0.5 $\log_{10}$ CFU per ml. This is nothing new to the *Campylobacter* community and underscores the need for a more reliable alternative method for quantification of live thermophilic *Campylobacter*. Due to provided reagents in the interlaboratory ring trial, variation might have been lower than actual, but many other aspects were variable and in the comparative study part two labs usd different reagents. While the reference method only enumerates culturable thermophilic *Campylobacter* under the laboratory conditions provided (CFU), the v-qPCR method quantifies all viable cells, which we defined as intact and potentially infectious units (IPIU) (Krüger et al., 2014). IPIU equals CFU plus VBNC. Especially under stressed conditions *Campylobacter* cells often do not grow on the plates, but may be reactivated under different growth conditions (Wulsten et al., 2020) or in the host model (Baffone et al., 2006; Cappelier et al., 1999; Chaisowwong et al., 2012). We, therefore, did not only compare the results from the alternative method against the CFU obtained from the samples but also from CFU determined from the spiking solution (without matrix stress exposure) within the international ring trial. The spiking solution was produced with *C. jejuni* cells in exponential growth phase and that these cryovials were homogeneous and stable according to ISO 13528:2015 (NRL for Campylobacter, 2021).

With the reference method for CFU determination from samples, we observed a positive bias of the values obtained from the alternative method. On average, this bias was 0.22 $\log_{10}$ per ml during our trueness study, no detectable systematic bias during the accuracy study and again a positive bias during the ring trial of the mean of maximal 0.5 $\log_{10}$ per ml. The bias seems to originate from under-determination of live *Campylobacter* spp. (CFU $\leq$ IPIU) by the reference method and was smaller using direct CFU determination of the spiking solution. The latter values display the maximal number of CFU in the sample (Figs. 5 and 6). Independent of the actual number of live *Campylobacter* in the spiking cryoculture solution, one would expect that the number of CFU detected by the reference method scales with the dilution level, which was poorly the case in particular at higher spiking levels (Figs. 5 and 6). Thus, we have to conclude that the alternative v-qPCR method is more reliable in detection of live *Campylobacter* spp. in meat rinses, since it is unbiased by variance in displaying CFU capacity. All criteria from ISO 16140-2:2016(E) were fulfilled for both the method comparison study and the interlaboratory study and therefore, the alternative v-qPCR method can be applied for the quantification of live thermophilic *Campylobacter* spp. in raw meat rinses for the tested range of contamination. Moreover, it is likely that the alternative v-qPCR performs better than the reference method (Table 4 and the reference method ISO 10272-2:2017).

Current risk assessment and legal consequences, e. g. the process hygiene criterion for *Campylobacter* spp. on chicken skin at slaughterhouse, are based on CFU data. For future application of the novel v-qPCR method and for adaptation of legal consequences, further studies should evaluate the bias of CFU underestimation dependent on sample type. For slaughterhouse samples, this bias is expected to be low, since CFU is supposed to correlate well with IPIU. For retail samples or other samples, in which *Campylobacter* spp. have been confronted with stress (e. g. raw milk, retails meat), the bias will be higher, improving current risk assessment and revealing underestimated transmission routes.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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