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Internal sample process control improves cultivation-independent quantification of thermotolerant *Campylobacter*

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ABSTRACT

Quantification of *Campylobacter* is challenging and one major reason is the fact that bacteria lose cultivability due to cold or oxygen stress during storage at retail. Alternative live/dead discriminatory qPCR currently lacks standardization and might overestimate live cells in the presence of dead cells.

In this study an internal sample process control (ISPC) was developed. The ISPC consists of a specified number of peroxide-killed *C. sputorum* cells to be added to each sample in order to monitor (i) the level of reduction of the signal from dead cells and (ii) DNA losses during sample processing. A species-specific fragment of the 16S rRNA gene of *C. sputorum* was selected as real-time PCR target, based on its similar size and gene copy number compared to the *C. jejuni/coli/lari* target and confirmed in an exclusivity study. Extension of the amplification oligonucleotides for the target of thermotolerant *Campylobacter* improved real-time PCR efficiency, rendering the method suitable for quantification according to international standards. Concordant PCR signal variation of both *C. jejuni* and *C. sputorum* targets in co-inoculated chicken rinses verified the suitability of the ISPC. This provides a crucial step towards implementation of cultivation-independent quantification for improved food safety of fastidious bacteria.

1. Introduction

Campylobacter is the major zoonotic agent in the European Union since 2005, causing 246,307 reported campylobacteriosis cases in 2016 (EFSA and ECDC, 2017). Around one third of the cases can be directly attributed to handling, preparation and consumption of broiler meat whereas up to 80% are ascribed to the “chicken reservoir as a whole”, for which further transmission routes remain unclear (EFSA, 2011). Due to high prevalence of *Campylobacter* on broiler meat, control measures focus on a quantitative reduction of the pathogen. Thus, rinse samples from broiler meat and skin are currently analysed by a time- and labor-consuming enumeration method (ISO_10272-2:2017). Efficiency of cultivation-dependent quantification is influenced by growth conditions and the physiological state of the bacteria impacting their ability to grow on a plate as colony forming units (CFU) (Amann et al., 1995; Krüger et al., 2014; Pinto et al., 2015; Tholozan et al., 1999). Moreover, fastidious organisms like *Campylobacter* lose cultivability due to cold or oxygen stress while poultry meat is stored at retail (El-Shibiny et al., 2009). These cells, although potentially infectious, are not detected as CFU, which leads to underestimating the risk of poultry meat contamination (Botteldoorn et al., 2008; Chaisowwong et al., 2012; Stingl et al., 2012). This highlights the need for cultivation-independent quantification methods. Alternative methods for the detection of bacterial life include pre-enrichment of samples prior to real-time PCR, detection of metabolic activity like membrane potential, rRNA content and membrane integrity (Fittipaldi et al., 2012; Nebe-von-Caron et al., 2000). The latter is used by live/dead discriminatory real-time PCR.

For detection of thermotolerant *Campylobacter* a fragment of the 16S rRNA gene is currently detected in diagnostic laboratories (ASU § 64, 2013; Josefsen et al., 2010). However, this real-time PCR is used for screening of positive samples, from which isolation of live *Campylobacter* is mandatory in order to comply with food regulations. The method was upgraded for live/dead discrimination (Josefsen et al., 2010; Krüger et al., 2014) and combined with a pretreatment of the sample with DNA-intercalating dyes (e.g. propidium monoazide, PMA) (Nocker et al., 2006, 2007, 2009). The dyes enter membrane-

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compromised cells, intercalate into cytoplasmic DNA and upon light exposure crosslink to DNA, inactivating it for PCR amplification (Nocker et al., 2006). Hence, treatment of the samples with PMA prior to real-time PCR quantifies cells that harbor an intact membrane, which we previously named intact and putatively infectious units (IPIU), comprising CFU and viable-but-non-culturable (VNBC) cells (Krüger et al., 2014).

The live/dead discriminatory real-time PCR has been applied to the detection and quantification of various bacterial species in clinical diagnostics (Rogers et al., 2008), water safety monitoring (Ditommaso et al., 2014; Seidel et al., 2017; Yanez et al., 2011) and in biofilms (Magajna and Schraft, 2015; Tavernier and Coenye, 2015). The method has also been applied in the determination of survival of probiotics (Ganesan et al., 2014; Kramer et al., 2009) and in food safety monitoring for quantitative detection of pathogenic and non-pathogenic bacteria such as *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Listeria innocua*, *Salmonella* and *Campylobacter* (Banihashemi et al., 2012; Elizaquivel et al., 2012; Josefsen et al., 2010; Lovdal et al., 2011).

However, limitations exist and the most important one relates to an insufficient reduction of the signal from dead cells (Banihashemi et al., 2012; Fittipaldi et al., 2012; Krüger et al., 2014). Hence, it is still unclear, how to interpret in detail the observed differences between cultural and PMA-qPCR enumeration results for *Campylobacter* on chicken carcasses during processing (Pacholewicz et al., 2013), for samples obtained from primary production (Seliwiorstow et al., 2015) or while evaluating the effect of poultry decontamination measures (Duarte et al., 2015). Signal reduction from dead cells depends on various parameters such as incubation temperature, type and concentration of dye, efficiency of the covalent crosslinking dye, light source, heterogeneity of the matrix and size of the target sequence (Fittipaldi et al., 2012; Krüger et al., 2014).

We proposed that this variability should be monitored by inclusion of an appropriate dead cell standard, which can be processed as internal sample process control (ISPC). This sample control is also important to calibrate to an absolute amount of genomic equivalents and to consider DNA losses during sample processing (Rossmanith et al., 2011; Zhang and Ishii, 2018).

Therefore, the aim of the study was the development of an internal sample process control (ISPC) with high similarity to the target. This ISPC should be used for quantitative detection of live *Campylobacter* by real-time PCR, serving as a reference standard with a specified number of dead *Campylobacter* to monitor the reduction of the signal from dead cells and the putative DNA losses during sample processing.

2. Materials and methods

2.1. Strains and growth conditions

C. jejuni DSM 4688 and *C. sputorum* DSM 5363 from −80 °C cryobank stocks (Mast Diagnostica, Germany) were cultured on Columbia blood agar (Oxoid, Germany) supplemented with 5% sheep blood (Oxoid, Germany) under microaerobic conditions (5% O_2, 10% CO_2, rest N_2). *C. jejuni* was initially cultured for 24 h at 42 °C and *C. sputorum* for 48 h at 37 °C. Both strains were subcultured for another 24 h at 42 °C or 37 °C, respectively.

2.2. Production of dead cells

After growth bacterial cells were resuspended in PBS at an OD_{600nm} of 0.2 (corresponding to ~10^9 bacterial counts/ml) and treated with 5% H_2O_2 for 1 h at room temperature as previously described (Krüger et al., 2014). After inactivation, cells were centrifuged at 16.000 × g for 5 min resuspended in the same volume of PBS and kept on ice. Inactivation was checked by absence of growth of a 10 µl loop of cell suspension on ColbA (~10^7 bacteria).

2.3. Preparation of chicken rinses and spiking of dead cells (C. jejuni and ISPC)

Skins and meat samples from *Campylobacter* negative chickens were rinsed in variable volumes of PBS (135 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) ranging from 1:3 to 1:10 of v/w of the sample. The samples were either rinsed manually or homogenized using a stomacher for 1–4 min. These variations guaranteed the preparation of chicken rinses with various contents of organic material, simulating heterogeneous matrices, which laboratories may encounter during routine work. In total 64 rinses ranging from 3 to 230 mg wet weight were prepared and analysed in two laboratories. Each chicken rinse sample of 1 ml was spiked in duplicate with 10^6 H_2O_2-treated dead *C. jejuni* and 10^6 H_2O_2-treated dead *C. sputorum* cells. In addition matrix-free controls (i. e. 1 ml of PBS inoculated with *C. jejuni* and *C. sputorum* in the same way as the rinse samples) and negative controls of the matrices were included.

2.4. PMA staining protocol and DNA extraction

For each sample, one of the duplicate was stained with PMA (Biotium Inc. USA) as described previously (Krüger et al., 2014). Briefly, samples were pre-incubated for 10 min at 30 °C before addition of 50 µM PMA and further incubation in the dark for 15 min at 30 °C and 700 rpm. Subsequently, photoactivation of PMA was performed using the PhAST blue system (GenIUL, Spain) for 15 min at 100% light intensity at room temperature. Afterwards samples were centrifuged for 5 min at 16,000 × g and stored at −20 °C until DNA extraction. DNA was extracted with the GeneJET Genomic Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions, eluted in 100 µl of buffer and analysed by real-time PCR on the same day.

For the specificity experiments the DNA of field strains was extracted by resuspension of the cell pellet in 5% Chelex 100 resin (Bio-Rad Laboratories GmbH, Germany), followed by incubation for 15 min at 95 °C and subsequent centrifugation. DNA of reference strains was extracted using the GeneJET Genomic Kit (Thermo Fisher Scientific, USA) or the Easy-DNA Kit (Thermo Fisher Scientific, USA). DNAs of field and reference strains were obtained during routine analysis in the national reference laboratory at BfR. The quality and quantity of the DNA was controlled by routine real-time PCR (ASU § 64, 2013) or other suitable multiplex PCR approaches as well as 16S rRNA sequencing (Chaban et al., 2009; Coenye et al., 1999; Houf et al., 2000; Wang et al., 2002; Weisburg et al., 1991).

2.5. Determination of the target sequence for the ISPC

Sequences were either obtained as GeneBank or NCBI reference sequences from the National Center for Biotechnology Information (NCBI, Bethesda MD, USA) or Sanger-sequenced using the primers 16SrRNA-F1 (5′-AGAGTTTGATCCTGGCTGAG-3′) and 16SrRNA-R1 (5′-AAGGAGGTGATCCAGCCGCA-3′) after amplification of a 1505 bp sized fragment of the 16S rRNA gene (Coenye et al., 1999). Sequence alignment was performed using the software MegAlign Pro 14.0 (DNASTAR, package MAUVE). For this purpose, the potential ISPC target was aligned with sequences of *C. jejuni*, *C. coli* and *C. lari* originating from reference and field isolates to design primers and the probe for the ISPC real-time PCR (Fig. 1). The aligned sequences were the following: *C. coli* BfR-CA-13310, *C. coli* ATCC 49941 (GeneBank: AY621115.1), *C. lari* BfR-CA-14811, *C. lari* DSM 11375 (NCBI Reference Sequence: NR_117762.1), *C. jejuni* BfR-CA-13311, *C. insulaenigrae* DSM 17739 (Sanger-sequenced), *C. jejuni* NCTC 11168 (NCBI Reference Sequence: NC_002163.1), *C. jejuni* ATCC 49943 (GeneBank: AY621112.1), *C. sputorum* RM3237 (NCBI Reference Sequence: NZ_CP019682.1), *C. sputorum* DSM 5363 (Sanger-sequenced).

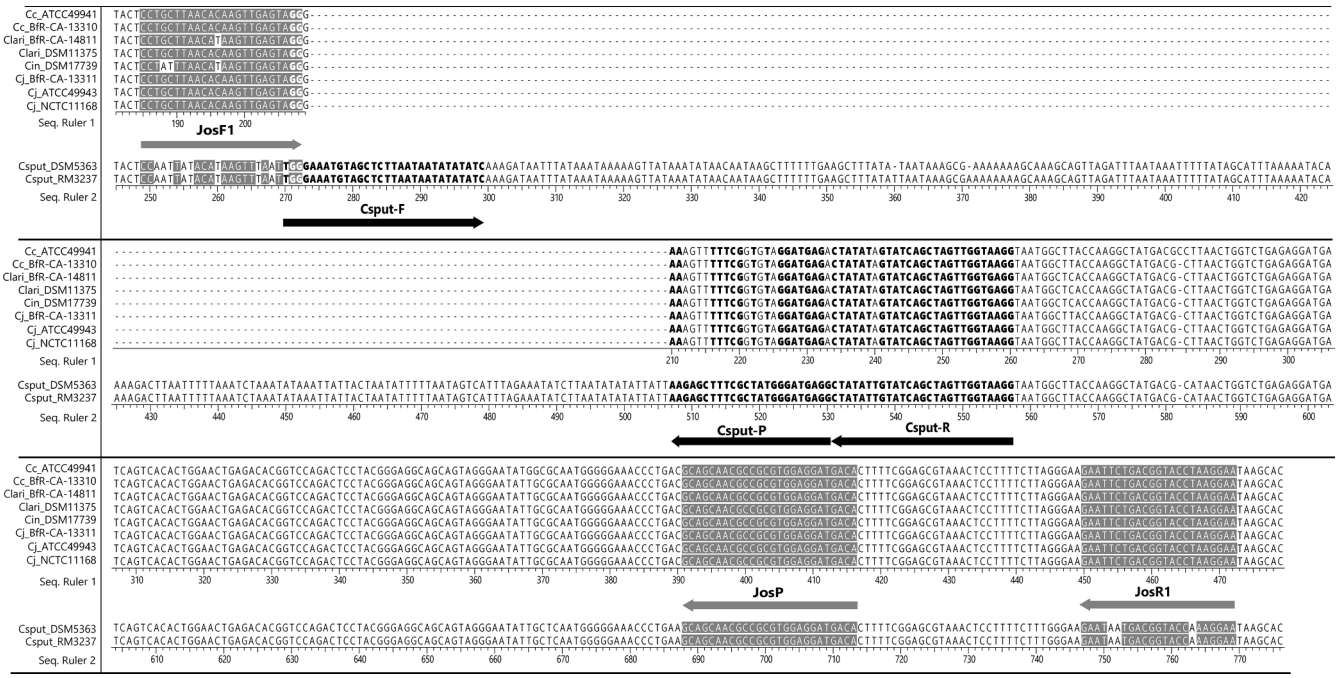

Fig. 1. Real-time PCR targets within the 16S rRNA genes of *C. jejuni* and *C. sputorum* (ISPC) were defined by sequence alignment of 16S rRNA gene fragments of *C. jejuni* (Cj), *C. coli* (Cc), *C. lari* (Clari), *C. insulaenigrae* (Cin) and *C. sputorum* (Csput). The figure indicates the primer and probe annealing sites for the thermotolerant *Campylobacter* (Jos-F1/R1/P; grey arrows) and the ISPC (Csput-F/R/P; black arrows) target. Sequence ruler 1 indicates the position of primers and probe in *C. jejuni* NCTC 11168 (NC_002163.1, Cjr01 (16S rRNA)), whereas sequence ruler 2 illustrates the position of primers and probe in *C. sputorum* RM3237 (NZ_CP019682.1, CSPUT_RS02990 (16S rRNA)). Homologous sequences of the Jos-F1/R1/P primers and probe is highlighted in grey; for the Csput-F/R/P set homologous sequences are marked in bold.

2.6. DNA genomic standards

The quantitative DNA standards for *C. jejuni* and the ISPC were based on genomic DNA, stabilized using DNAstable protectant (Biomatrica, San Diego, CA) and stored at room temperature in the dark. The quality of the DNA was evaluated by spectral analysis (NanoDrop Spectrophotometer, Thermo Fisher Scientific, USA) and the concentration was fluorimetrically quantified by Qubit 3.0 Fluorometer (Q32851, dsDNA HS Assay Kit 0.2–100 ng; Thermo Fisher Scientific, USA). Based on the size of the genomic DNA of *C. jejuni* NCTC 11168 of 1.6 Mb, it was estimated that 5.94×10^5 genomic copies are present in 1 ng of genomic DNA (Krüger et al., 2014). Since only relative quantitative data of the *C. sputorum* target are needed, the same calculations were applied for production of the *C. sputorum* genomic standard, assuming a similar sized genome. Likewise, aliquots of 16.8 ng of genomic DNA (i. e. 10^7 genomic copies) were stabilized and exchanged between laboratories.

2.7. Real time PCR quantification

Three different duplex PCR reactions were performed for (i) the detection of a fragment of the 16S rRNA of *C. jejuni* or (ii) *C. sputorum* or (iii) the *mapA* gene of *C. jejuni*. Real-time PCR was done on an ABI Prism 7500 (Life Technologies, USA) in Laboratory 1, whereas Laboratory 2 used an AriaMx Fast Cycler (Agilent Technolgies, USA). For each real-time PCR target, appropriate DNA standards of genomic DNA, covering five 10-fold dilutions (freshly diluted in the background of 100 ng/µl salmon sperm DNA (AppliChem GmbH, Germany; or Thermo Fisher Scientific, USA)), ranging from 5 to 5×10^4 genomic copies were applied in duplicates in each real-time PCR assay.

2.7.1. 16S rRNA C. jejuni target

The real-time PCR reaction of the 288 bp fragment of *C. jejuni* was performed as follows. 25 µl of PCR reaction mixture consisted of 1 U Platinum Taq DNA Polymerase (Life Technologies, USA), 1 × reaction buffer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP (Thermo Fischer Scientific, USA), ROX passive reference dye (Life Technologies, USA), 500 nM of Jos-F1 (5′-CCTGCTTAACACAAGTTGAGTAGG-3′) and Jos-R1 (5′-TTCC TTAGGTACCGTCAGAATTC-3′), and 100 nM of the dark-quenched hydrolysis probe JosP (6FAM-TGTCATCCTCCACGCGGCGTTGCTGC-BBQ) (BBQ, BlackBerry quencher, TIB MOLBIOL, Germany). As internal amplification control (IAC), 25 copies of the IPC-ntb2 detecting a 125-bp sequence of the rbcMT-T gene from *Nicotiana tabacum* with 300 nM forward (5′-ACCACAATGCCAGAGTGACAAC-3′) and reverse primer (5′-TACCTGGTCTCCAGCTTTCAGTT-3′) and 100 nM of the dark-quenched hydrolysis probe (TAMRA- or HEX-CACGCGCATGAAGTTAGGGG ACCA-BBQ) was used (Anderson et al., 2011). In Laboratory 2 the mastermix components were substituted by the QuantiTect Multiplex PCR NoRox mastermix (Qiagen, Germany).

2.7.2. 16S rRNA C. sputorum target

In a separate real-time PCR reaction, a 286 bp fragment of the 16S rRNA gene of *C. sputorum* DSM 5363 was detected. The PCR master mix was the same as mentioned in 2.7.1. but contained instead of Jos-F1/R1/P primers and probe, 500 nM of the forward primer Csput-F (5′-TGGGAAATGTAGCTCTTAATAATATATATC-3′), 500 nM of the reverse primer Csput-R (5′- CCTTACCAACTAGCTGATACAATATAG-3′) and 100 nM of the dark-quenched probe Csput-P (Cy5-CCTCATCCCAT AGCGAAAGCTCTT-BBQ). For both targets of *Campylobacter* 16S rRNA gene, initial denaturation was conducted in Laboratory 1 for 3 min at 95 °C, followed by 45 cycles of 15 s at 95 °C, 1 min at 60 °C, and 30 s at 72 °C, whereas in Laboratory 2 initial 15 min at 95 °C was followed by 45 cycles of 30 s at 95 °C and 1 min at 60 °C.

2.7.3. mapA target of C. jejuni

The detection of a 95 bp fragment of the *C. jejuni mapA* gene was performed as described before (Best et al., 2003; Mayr et al., 2010), using the same PCR master mix as described above but substituting the

Table 1
Results of inclusivity and exclusivity test using the modified primer set Jos-F1 and Jos-R1.

Tested reference strains	results	Tested field isolates	Number of isolates	results
Arcobacter butzleri DSM 8739	negative	*Arcobacter butzleri*	10	negative
Arcobacter cryaerophilus DSM 7289	negative	*Arcobacter cryaerophilus*	1	negative
Arcobacter skirrowii DSM 7302	negative	*C. coli*	40	positive
C. coli DSM 4689	positive	*C. jejuni*	63	positive
C. concisus DSM 9716	negative	*C. lari*	34	positive
C. concisus CCUG19996	negative	*C. upsaliensis*	2	negative
C. curvus CCUG13146	negative	*Helicobacter pullorum*	5	negative
C. fetus fetus DSM 5361	negative			
C. gracilis DSM 19528	negative			
C. hyointestinalis DSM 19053	negative			
C. insulaenigrae DSM 17739	positive			
C. jejuni DSM 4688	positive			
C. lari concheus LMG 11760	positive			
C. lari DSM 11375	positive			
C. lari lari LMG 8846	positive			
C. lari CCUG 18294	positive			
C. lari CCUG 19512	positive			
C. lari CCUG 23947	positive			
C. lari LMG 19453	positive			
C. lari concheus LMG 23918	positive			
C. peloridis LMG 23910	positive			
C. peloridis LMG *17564*	positive			
C. sputorum CCUG 37580	negative			
C. upsaliensis DSM 5365	negative			
C. sputorum bubulus DSM 5363	negative			
C. sputorum sputorum DSM 10535	negative			
C. upsaliensis CCUG 33880	negative			
Staphylococcus aureus DSM 1104	negative			

Table 2
Results of the exclusivity study of the ISPC real-time PCR.

Tested reference strains	results	Tested field isolates/matrices	Number of isolates/matrices	results
Arcobacter butzleri DSM 8739	negative	*C. jejuni*	108	negative
Arcobacter cryoaerophilus DSM 7289	negative	*C. coli*	63	negative
Arcobacter skirrowii DSM 7302	negative	*C. lari*	1	negative
C. coli DSM 4689	negative	*C. lanienae*	1	negative
C. concisus CCUG 19996	negative	*Helicobacter pullorum*	2	negative
C. concisus DSM 9716	negative	Chicken rinse	11	negative
C. curvus CCUG 13146	negative	Raw milk (enrichment)	1	negative
C. fetus fetus DSM 5361	negative			
C. gracilis DSM 19528	negative	*C.jejuni* BfR-CA-15078 (field strain, Vietnam)	1	negative
C. hyointestinalis DSM 19053	negative	*C. coli* BfR-CA-15062 (field strain, Vietnam)	1	negative
C. insulaenigrae DSM 17739	negative			
C. jejuni dolei LMG 8843	negative			
C. jejuni DSM 4688	negative			
C. lari concheus LMG 11760	negative			
C. lari DSM 11375	negative			
C. peloridis LMG 13910	negative			
C. sputorum bubulus DSM 5363[a]	positive			
C. sputorum sputorum DSM 10535[a]	positive			
C. upsaliensis DSM 5365	negative			
C. upsaliensis CCUG 33880	negative			

[a] Positive controls for ISPC.

Jos-F1/R1/P primers and probe with 300 nM of the forward (5′-CTGG TGGTTTTGAAGCAAAGATT-3′) and reverse (5′-CAATACCAGTGTCTA AAGTGCGTTTAT-3′) primers and 100 nM of the dark-quenched probe (FAM-TTGAATTCCAACATCGCTAATGTATAAAAGCCCTTT-BBQ) for *mapA* detection (Best et al., 2003; Mayr et al., 2010).

2.8. Determination of LOD_{95}

A limit of detection (LOD) for qPCR was determined in two steps. First, an LOD_6 screening was performed, which is defined by the genomic DNA amount leading to a specific positive amplification signal for all six replicates tested. For this purpose, DNA dilution series of *C. sputorum* DSM 5363 and *C. jejuni* NCTC 11168 ranging from 20 to 1 copy were tested in two independent real-time PCR runs. The target DNA was diluted in 100 ng/µl salmon sperm background DNA (AppliChem GmbH, Germany; or Thermo Fisher Scientific, USA). In an extended experiment, the LOD_{95} was determined over 60 replicates to validate the LOD_6 results (CLSI, 2004). This is defined as the LOD, at which the analytical assay detects the presence of the analyte with at least 95% confidence interval, thus ensuring ≤5% false negative results.

3. Results

3.1. Development of a novel real-time PCR for detection of an appropriate ISPC target

For detection of thermotolerant *Campylobacter* a fragment of the 16S

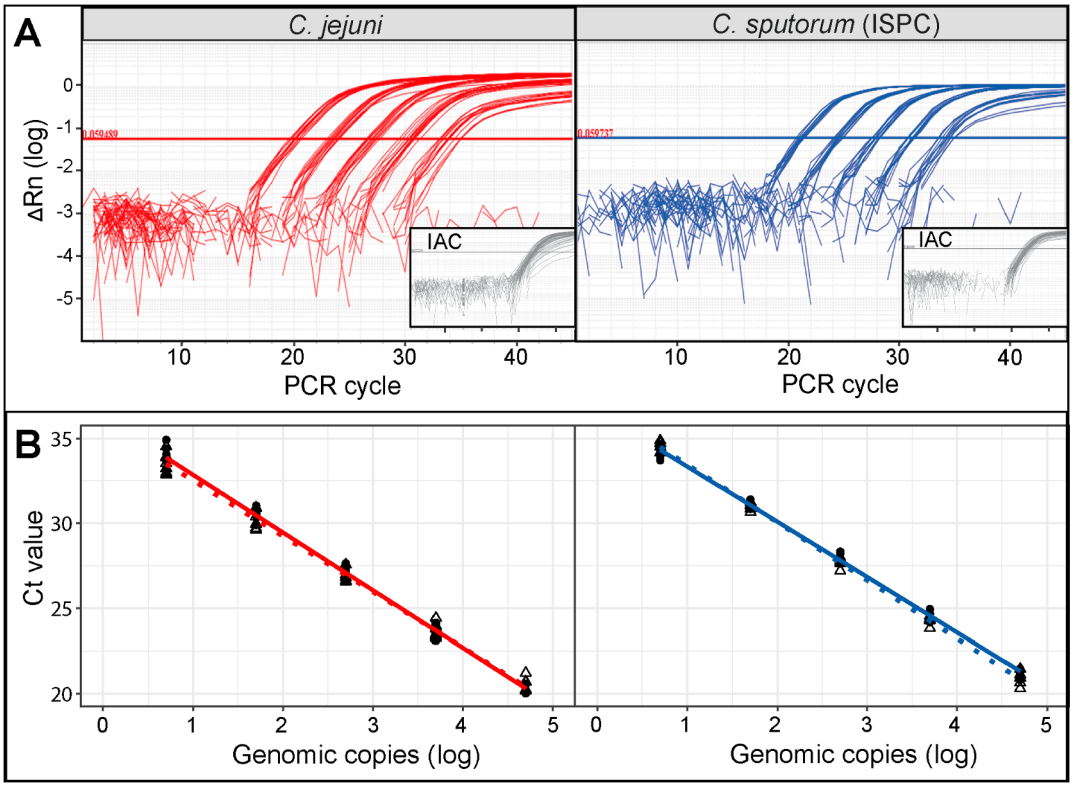

Fig. 2. Quantitative real-time PCR targeting *C. jejuni* and *C. sputorum* are highly reproducible using genomic standards. A, Real-time PCR amplification curves of genomic standards (decimal dilutions from 50.000 to 5 copies) in two duplex PCR reactions (*C. jejuni* (red curves) or *C. sputorum* (ISPC, blue curves) each with internal amplification control (IAC, grey curves in insert plots)). ΔRn, fluorescence signal of reporter minus baseline in arbitrary units (log scale). B, Quantitative comparison of independently produced batches of genomic standards (n ≥ 6) in *C. jejuni* and *C. sputorum* real-time PCR runs performed in two different laboratories (closed circles and solid line, Laboratory 1; open triangles and dotted line, Laboratory 2).

rRNA gene is currently detected in diagnostic laboratories (ASU § 64, 2013). Since the 16S rRNA gene is essential in bacteria, construction of a genetically modified organism, lacking the target gene, was not considered. Instead, a search for significant variation of the 16S rRNA gene at the annealing sites of the forward and reverse primers of the routine real-time PCR assay revealed that the related non-thermotolerant atypical *Campylobacter sputorum*, isolated from sheep and cattle, also possesses three copies of the 16S rRNA gene and carries an intervening sequence at in the 16S rRNA helix 11 region (Iraola et al., 2014; Tazumi et al., 2010). This region was targeted for the design of two primers and a probe for an independent real-time PCR. Fig. 1 illustrates the aligned sequences of *C. jejuni*, *C. coli, C. lari* and *C. sputorum* 16S rRNA gene fragments from reference and field isolates. A real-time PCR assay was designed to match the fragment length of the *C. jejuni* target (Fig. 1), with maximal sequence distinction. Likewise, the forward Jos-F1 primer for detection of thermotolerant *Campylobacter* exhibits 9 mismatches, while the reverse primer Jos-R1 contains 3 bp mismatches in comparison to the *C. sputorum* sequence (Fig. 1 and see below). Results from the exclusivity study revealed lack of a false-positive amplification result (100% specificity) in all 209 tested samples as summarized in Table 2.

3.2. Efficiency and LOD_{95} of the real-time PCRs

To increase the efficiency of the real-time PCR for thermotolerant *Campylobacter*, the original primers used by Josefsen et al. (2010) were slightly modified resulting in an increased theoretical annealing temperature around 3 °C (http://www.oligoevaluator.com/OligoCalcServlet) and were named Jos-F1 and Jos-R1. The modified primer set was evaluated for inclusion of thermotolerant *Campylobacter* and for exclusion of other bacteria using 155 field strains including *Campylobacter* (139), *Arcobacter* (11) and *Helicobacter pullorum* (5) as well as 28 reference strains of Campylobacterales (Table 1). The reference strain *C. insulaenigrae* DSM 17739, known for its similarity towards *C. jejuni* (Gonzalez et al., 2011), was tested positive. The alignment of the *C. insulaenigrae* DSM17739 16S rRNA gene sequence and the utilized primer pair Jos-F1 and Jos-R1 confirmed a high similarity with only 3 mismatches in Jos-F1 (Fig. 1). When the original primer set (Josefsen et al., 2010; Lübeck et al., 2003) was applied the strain amplified as well. Therefore, the specificity of the slightly modified oligonucleotides was not changed compared to the original study. However, this modification increased PCR efficiency, as compared with previous observations using the original oligonucleotides (Josefsen et al., 2010; Krüger et al., 2014). The average efficiency of the real-time PCR using Jos-F1/R1/P for *C. jejuni* DSM 4688 genomic DNA was 96 ± 5% ($0.991 \leq r^2 \leq 1.00$; n = 56), while with the previous oligos the efficiency was confirmed to be lower with 88 ± 3%, $0.995 \leq r^2 \leq 1.00$; n = 11. The real-time PCR with Csput-F/R/P on *C. sputorum* DSM 5363 genomic DNA displayed an efficiency of 99 ± 5% ($0.983 \leq r^2 \leq 1.00$; n = 34).

A prerequisite for a reliable (absolute) quantification of target DNA in independent laboratories is the production and preservation of appropriate standard DNA, containing distinct amounts of the target sequence. Results of DNA standards prepared from *C. jejuni* and *C. sputorum*, fluorimetrically quantified, stabilized, stored at room temperature for maximum one year and exchanged between the two laboratories showed similar and reproducible standard curves for both targets. The standard deviations of the log translated Ct value within a run caused by independent lots of genomic standards did not exceed log 0.2 genomic copies in both laboratories and both targets (Fig. 2).

The limit of detection (LOD) was determined by analysis of 1, 2 and 3 genomic DNA copies in 60 replicates each. LOD_{95} of the Jos-F1/R1/P

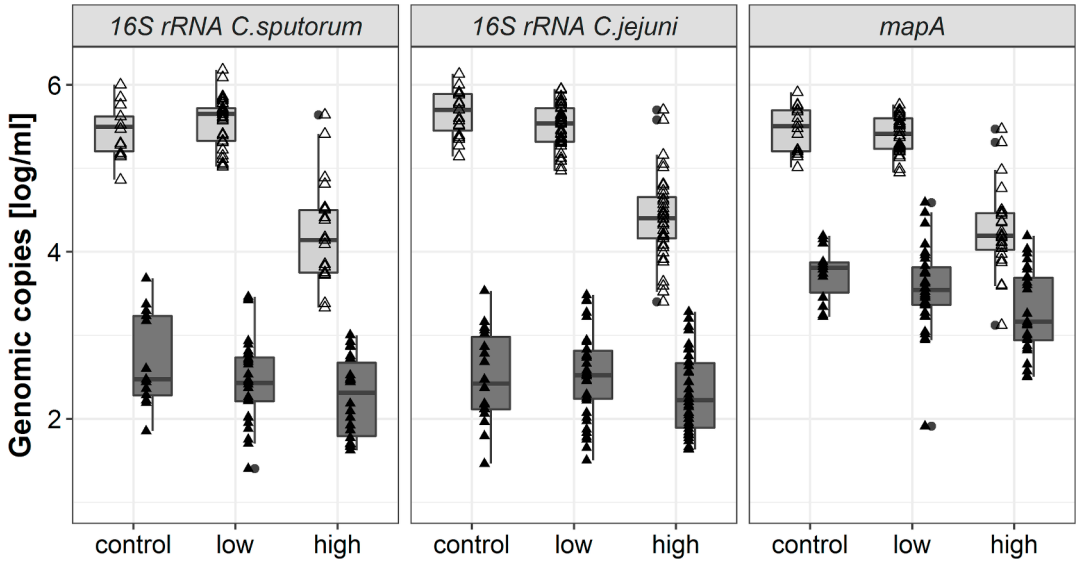

Fig. 3. High sample-to-sample variability of the PCR results of similarly spiked chicken rinses substantiates the need for parallel ISPC detection. Log genomic copies of total DNA without PMA treatment (light grey and open symbols) and of the remaining signal from dead cells in the presence of PMA (dark grey and closed symbols) obtained from chicken rinses and matrix-free controls, dually spiked each with 10^6 dead *C. jejuni* and *C. sputorum*. 16S rRNA gene targets of both species and the *mapA* gene of *C. jejuni* were quantified by real-time PCR; chicken matrices were categorized in organic load below (low) and above (high) 100 mg wet weight. The boxplot length corresponds to the interquartile range (IQR) of data (50% of the data), the horizontal bar indicates the median value; black 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. Real-time PCR values below LOD_{95} were omitted from analysis.

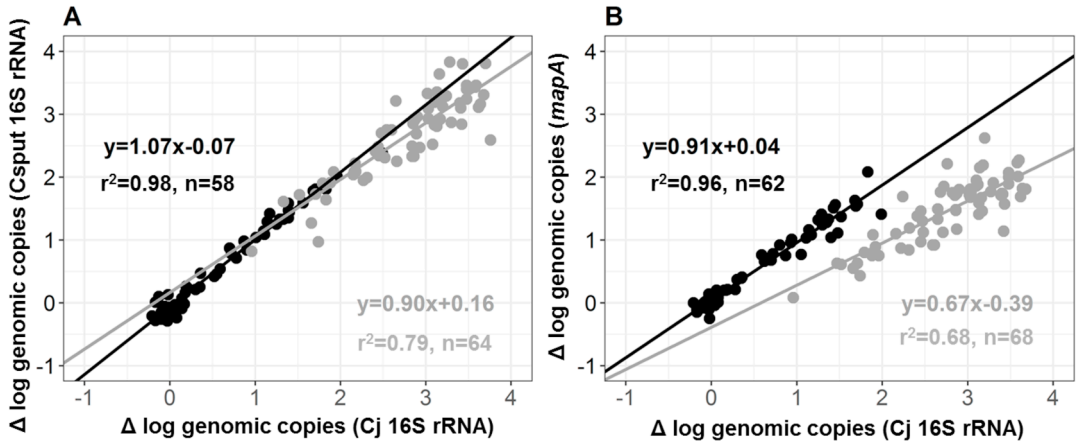

Fig. 4. Sample-to-sample variability of the *C. jejuni* 16S rRNA signal is most reflected by parallel monitoring of *C. sputorum* 16S rRNA target (ISPC). Reanalysis of data from Fig. 3. Δlog copies upon PMA treatment (in grey) and due to DNA loss (in black) were computed and analysed by regression. The slope and intercept are given in the equations, with r^2 (correlation coefficient) and n, number of samples. A, correlation of the 16S rRNA gene targets of *C. jejuni* and *C. sputorum*; B, correlation of the 16S rRNA gene target and the *mapA* target of *C. jejuni* in the same cell.

real-time PCR on *C. jejuni* NCTC 11168 genomic DNA and of the Csput-F/R/P real-time PCR on *C. sputorum* DSM 5363 was assessed to be each 2 genomic copies. This corresponds to 6 copies of the 16S rRNA target, since the *Campylobacter* genome carries three rRNA operons. For the completely sequenced *C. jejuni* NCTC 11168 LOD_{95} can be interpreted as absolute under the tested conditions. The *C. sputorum* standard is only used as a relative quantitative standard and LOD_{95} determination of this novel real-time PCR was intended to prove similar sensitivity and efficiency as compared to the thermotolerant PCR assay.

3.3. Variation in qPCR results of the ISPC mimics those of the target C. jejuni

Does the ISPC monitor the same variations during sample processing as the target bacterium? In order to answer this question, the two laboratories quantified each 10^6 of H_2O_2-inactivated *C. jejuni* and *C. sputorum* cells (ISPC) spiked in chicken rinses with variable wet organic matter content. The rationale behind was to produce all kinds of possible rinses and check that for each matrix the ISPC reflects the behaviour of the *C. jejuni* target. They should simulate different rinses laboratories produce during routine analysis. Fig. 3 summarizes the log genomic copies of each tested target in samples with variable load of wet organic matter content. Without any disturbance of the quantification method (DNA loss, different reduction of the signal of dead cells), it is expected that each sample analysis leads to the same result. However as shown in Fig. 3, variations of matrix led to considerable different absolute quantification of target DNA. As expected, DNA loss and insufficient reduction of the dead cell signal was most observed for matrices with high load of organic material. These observations confirm the need for standardization via application of the ISPC. In order to see if the application of the ISPC can serve for normalization of the measured target signal, the data from Fig. 3 were reanalyzed. Fig. 4

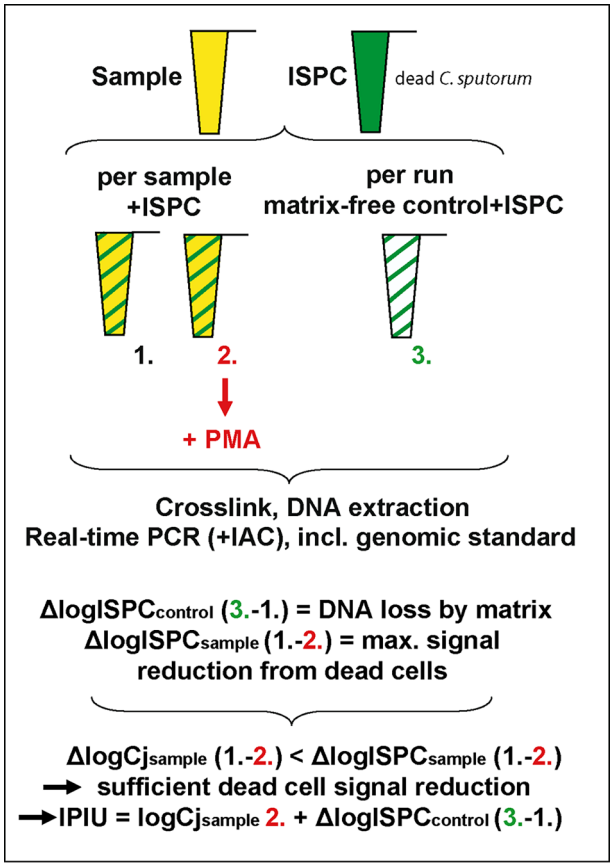

Fig. 5. Schematic overview of the application of the ISPC for quantification of live *C. jejuni* by real-time PCR. The ISPC (in green) is added to two aliquots of each sample (in yellow). Per run one matrix-free control with the ISPC is included (in white). One of the sample aliquots receives PMA (in red). The samples are crosslinked and DNA is extracted. The DNA is analysed in two duplex PCRs, with each containing the IPC-ntb2 plasmid as internal amplification control (IAC). From the results DNA loss by matrix effects and the maximal reduction of dead cell signal are estimated from the ISPC and used for calculation of intact and putatively infectious units of the target *C. jejuni* (IPIU).

illustrates Δlog copies due to PMA treatment (comparison of samples with and without treatment) and Δlog copies due to DNA loss (comparison of spiked cells in PBS with chicken rinse samples). Intriguingly, the quantified ISPC target, the 16S rRNA of *C. sputorum,* varied in a very similar way to the thermotolerant *Campylobacter* 16S rRNA target (Fig. 4A). In particular, DNA loss (y = 1.07× - 0.07, r^2 = 0.98, 95% confidence (slope ± 0.045 and intercept ± 0.040)); Fig. 4A, black symbols and lines) and reduction of the signal from dead cells (y = 0.90x + 0.16, r^2 = 0.79; 95% confidence (slope ± 0.117 and intercept ± 0.335); Fig. 4A, grey symbols and lines) correlated well between both targets. We also confirmed that the target size and number per genomic copy impacts on the correlation of qPCR variations due to matrix effects. For this purpose, a real-time PCR was applied to quantify a 95 bp fragment of the single copy *mapA* gene of *C. jejuni* (Fig. 4B). Although the *mapA* target is situated in the same cells as the 16S rRNA target of thermotolerant *Campylobacter*, reduction of signal from dead cells caused by treatment with PMA was underestimated (y = 0.67× - 0.39; 95% confidence (slope ± 0.110 and intercept ± 0.316)) and displayed only a correlation coefficient of r^2 = 0.68 (Fig. 4B, grey symbols and lines). As expected, DNA loss was detected in a similar way for *mapA* and the 16S rRNA target of thermotolerant *Campylobacter* (y = 0.91x + 0.04; r^2 = 0.96; 95% confidence (slope ± 0.049 and intercept ± 0.043)); Fig. 4B, black symbols and lines).

4. Discussion

4.1. Development of the ISPC

In this study we developed a dead cell standard (ISPC), which should prevent overestimation of live cells and monitor DNA loss due to extraction. An important aspect of the ISPC is that it shares characteristics with the target organism, but does not disturb its detection. Thus, the selected sequence of the presented ISPC was based on *C. sputorum* which was previously isolated from bovine semen (Iraola et al., 2014) and not found in poultry so far. Moreover, *C. sputorum* has an intervening sequence (IVS) in its 16S rRNA genes (Etoh et al., 1998; Tazumi et al., 2009, 2010), absent in thermotolerant *Campylobacter*. The amplified fragment has a similar size and matches the number of target copies per chromosome as compared to the *C. jejuni/coli/lari* target (Josefsen et al., 2010).

Several steps have been taken to validate the suitability of the *C. sputorum* dead cell standard as ISPC. Firstly, the absence of the IVS in 16S rRNA genes of thermotolerant *Campylobacter* species was confirmed by sequence alignment (Fig. 1) and by an exclusivity study performed on various Campylobacterales isolates, including reference strains and food matrices typical for *Campylobacter* (Table 2). In addition, we showed that the length of the fragment is crucial for signal suppression from dead cells (Fig. 4B), since the probability of PMA intercalation into the amplicon increases with length (Banihashemi et al., 2012).

Heterogeneous matrices may occur in practice while quantifying *Campylobacter* on poultry products and we showed that the ISPC is able to detect these variations and monitor DNA losses as well as insufficient signal reduction from dead cells (Fig. 4).

4.2. Real-time PCR efficiency and sensitivity

The real-time PCR efficiency was increased for detection of the 16S rRNA gene target in thermotolerant *Campylobacter* from 88% ± 3 to 96 ± 5% by slightly modifying the amplification primers for improved theoretical annealing temperature. The inclusivity and exclusivity study showed that this modification did not lead to alteration of specificity (Table 1).

The presented real-time PCR method enables the detection of up to 2 copies per PCR reaction, which correlates to 20 or 40 genomic copies in 1 ml of chicken rinse, depending on the maximal amount of DNA eluate applied as template in the PCR reaction (10 or 5%, respectively). In a previous study, it was estimated that in stationary phase, each *Campylobacter* cell contains one genomic copy (Krüger et al., 2014). Hence, the real-time PCR presents sufficient sensitivity to be applied to quantification of 20–40 viable cells/ml of chicken rinse.

In order to guarantee that independent laboratories apply comparable standard curves, genomic DNA genomic standards were prepared. We showed that DNA standards independently produced led to comparable results with low maximal standard deviations of absolute Ct values (corresponding to log 0.2 genomic copies) (Fig. 2B).

4.3. How to apply the ISPC and how to interpret results?

How should the ISPC be applied in praxis? Per sample two aliquots are spiked with the ISPC and one of the two aliquots additionally receives PMA (Fig. 5). The ISPC alone is also included in the PCR setup, serving as a matrix-free control. After treatment/crosslinking, cells are pelleted and processed for DNA extraction and real-time PCR analysis. From the results DNA loss by matrix effects and the maximal reduction of dead cell signal are estimated from the ISPC and used for calculation of intact and putatively infectious units of the target *C. jejuni* (IPIU).

In principle, two results of the real-time PCRs can be obtained (Table 3). (i), the Δlog of the ISPC in the sample ($\Delta logISPC_{sample}$) ± PMA significantly exceeds the measured Δlog reduction of the target in the sample ($\Delta logCj_{sample}$) ± PMA. In this case, the

Table 3
Interpretation of results from PMA qPCR standardized by ISPC.

No.	If	then	conclusion
i	$\Delta logCj_{sample}$ [a)] < $\Delta logISPC_{sample}$ [b)]	$logCj_{sample+PMA}$ + $\Delta logISPC_{control}$ [c)] = $logCj_{live}$ (=IPIU)	☺ reliable results
ii	$\Delta logCj_{sample} \geq \Delta logISPC_{sample}$	quantified DNA exclusively stems from dead bacteria OR live bacteria are masked by dead bacteria OR inefficient dead cell signal reduction	☹ method is limited

a), $\Delta logCj_{sample}$ ($logCj_{sample} - logCj_{sample+PMA}$); b), $\Delta logISPC_{sample}$ ($logISPC_{sample} - logISPC_{sample+PMA}$); c), $\Delta logISPC_{control}$ ($logISPC_{control} - logISPC_{sample}$); Cj, *C. jejuni*.

method worked well and the log target bacteria quantified in the presence of PMA can be adjusted for eventual DNA loss. This is done by addition of the Δlog difference between the $logISPC_{control}$ in the matrix-free control and the $logISPC_{sample}$ ($\Delta logISPC_{control}$), leading to calculation of the number of intact and potentially infectious units (IPIU) (Fig. 5, IPIU = $logCj_{sample}$ from tube 2. + $\Delta logISPC_{control}$ (3.-1.)).

In case the $\Delta logISPC_{sample}$ of the ISPC ± PMA is smaller than or equal to the measured $\Delta logCj_{sample}$ reduction of the target ± PMA, either only dead target bacteria are present or the viable bacteria are masked by too many dead bacteria or inefficient dead cell signal reduction is detected.

5. Future aspects

We expect that the method will be useful for praxis application at all stages of the poultry food chain. Especially at retail, where the current enumeration method apparently does not reflect the real risk to the consumer (Stingl et al., 2012, 2015) and at farm level for improved detection of transmission routes, such novel approaches are needed.

In our proof-of-principle study we added a relatively high amount of dead *C. jejuni* and *C. sputorum* (ISPC). This ensured that also in the presence of PMA a Ct-value was detected for monitoring the PMA effect represented in Figs. 3 and 4. Such high concentrations of both targets will lead to competition in the real-time PCR, thereby, two separate duplex PCRs, each including an internal amplification control (IAC), were performed. In the future, this might be improved by applying a triplex PCR assay for *C. jejuni*, ISPC and IAC with lower ISPC concentration. This would increase practicability and decrease costs.

Furthermore, in order to prove that intact *Campylobacter* quantified by ISPC-controlled live/dead discriminatory qPCR really bear the potential of regrowth, resuscitation experiments are needed. This will help in demonstrating that intact and putatively infections units (IPIU) are a valuable parameter to improve food safety. In conclusion, *Campylobacter* can be viewed as a model organism for fastidious foodborne bacteria and the approach described here may be adopted for other organisms.

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