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Research Paper

Viable *Campylobacter jejuni* on Eggshells and Its Potential to Cross-contaminate Egg White and Yolk When Using a Manual Separation Technique, Determined by Culture and Propidium Monoazide (PMA) qPCR

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ABSTRACT

Manual separation of egg yolk from egg white using the eggshell is common practice in private households. For this, the egg is cracked and both components are separated by passing the egg yolk back and forth between the two halves of the eggshell, allowing the egg white to drip down while the egg yolk remains in the shell. During this process, the egg content naturally gets in contact with the outside of the eggshell, which might lead to a cross-contamination with its microorganisms, thus was correspondingly assessed in this study. *Campylobacter jejuni* is one of the most important zoonotic pathogens that can be found on eggshells. Therefore, this bacterium was used to artificially contaminate the eggshells (n = 22) with concentrations of 3.1 ± 0.6 $\log_{10}$ cfu/g. After separating the egg yolk from the egg white, cross-contamination was determined using culture and qPCR. Altogether, cross-contaminations with *C. jejuni* were found in 15 egg white (68%) and in three egg yolk (14%) samples. Afterward, 90 eggs from 30 egg packs from different producers in and around Munich (Germany) were obtained for field study purposes. To address the problem of culturing due to a possible viable but nonculturable (VBNC) status of *C. jejuni*, a method to differentiate viable and dead *C. jejuni* on eggshell using 10 μM propidium monoazide (PMA) and qPCR was developed. As a result, seven egg packs (23%) were positive for *C. jejuni*. Of these, only one (3%) was contaminated with viable cells, but still in a concentration of 3.3 $\log_{10}$ cells/g shell. According to these results and considering that eggshells might also be naturally contaminated with other pathogens, the authors recommend avoiding the manual separation technique of egg white and yolk by the eggshell. Especially if raw egg white or yolk is used for preparation of not sufficiently heated foods, where contaminating pathogens are not inactivated during processing, this technique might be a safety hazard for the consumer.

Since 2007 campylobacteriosis is the most frequently reported foodborne gastrointestinal infection in humans in the European Union (EU, EFSA, 2022). In 2021, the number of confirmed cases of human campylobacteriosis was 127,840, corresponding to an EU notification rate of 41.1 per 100,000 population (compared to salmonellosis, 15.7 per 100,000 population, EFSA, 2022). Contaminated food is by far the most common source of *Campylobacter* infections in humans. Especially relevant are undercooked chicken meat, but also pork, beef and other nonready to eat food, raw milk, and milk products, as well as contaminated drinking water (EFSA, 2022; Hyllestad et al., 2020). Infection through chicken eggs may occur through contaminated eggshells (BfR, 2018). In 88% of the reported campylobacteriosis cases, *C. jejuni* can be traced back as the outbreak-causing species (EFSA, 2022).

Campylobacter spp. are commonly present in the intestines of poultry. In Bavaria (Germany), 29% (n = 145/400) and 36% (117/399) of the laying hens from conventional and organic farming systems, respectively, were found to be positive for *Campylobacter* spp. (Schwaiger et al., 2008). A high prevalence was observed in Tunisia, where 42.3% (n = 155/366) of the laying hens carried these bacteria in their intestines (Gharbi et al., 2022). In Belgium, all investigated laying hens and breeding flocks (n = 20) were positive for *Campylobacter* spp. (Rasschaert et al., 2007). In organic laying hen farms in Finland, the prevalences of *Campylobacter* spp. ranged between 76% (n = 13/17) and 84% (n = 16/19), depending on the season (Sulonen et al., 2007). For the latter study, the percentage of positive samples within a positive flock varied between 5% and 100% (Sulonen

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et al., 2007). In agreement with the above-mentioned studies, *C. jejuni* is the most abundant species in chickens. Infected laying hens can regularly excrete large amounts of *C. jejuni* with their feces (Ahmed et al., 2013) and eggshells can be easily contaminated. Concerning eggshells´ contamination with *Campylobacter* spp., numerous studies have shown variable prevalences depending on housing system (e.g., battery, barn raising, free-ranging system) and region, as examples, 0.5% (n = 4/800, Schwaiger et al., 2008) or 0.4% (n = 11/2,710, Messelhäusser et al., 2011) in Germany; 5.8% (n = 7/120) in Poland (Gondek et al., 2013), 7.0% (n = 7/100) in Iran (Jonaidi-Jafari et al., 2016), 26.0% (n = 22/86) in Tunisia (Gharbi et al., 2022), and 4.1% (n = 16/392, Jones et al., 2016) to 16.8% (n = 59/352, Parisi et al., 2015) in the United States.

Eggs visibly contaminated with feces or any excrement are not allowed to be sold at retail. However, this contamination may not easily be seen. In addition, according to EU Commission Regulation (EC) No 589/2008, eggs sold at retail shall not be washed, because of a potential damage of the cuticle, which acts as a physical barrier of the eggshell. Such damage may favor trans-shell contamination with bacteria and moisture loss, increasing the risk for consumers, particularly if subsequent drying and storage conditions are not optimal (EU Commission Regulation (EC) No 589/2008).

Given that adequate heating or pasteurization can inactivate *Campylobacter* spp. in egg contents (Sato and Sashihara, 2010), the risk of campylobacteriosis mainly accounts for food containing raw or not properly cooked eggs and egg products such as sauces and dressings (e.g., mayonnaise, hollandaise, and aioli), desserts (e.g., tiramisu and mousse), or even drinks (e.g., raw egg protein drinks and eggnog). By preparing these foods in private households, contamination from eggshell to egg content, to other foods or to kitchen utensils, can occur by improper handling of raw eggs (BfR, 2018), for example, when the egg content gets in contact with the contaminating eggshell during cracking. A study conducted in commercial egg-breaking facilities in Japan showed that *Campylobacter* spp. could be isolated from 27.9% (n = 39/140) of unpasteurized liquid whole egg and 36.0% (n = 18/50) of unpasteurized liquid egg yolk samples (Sato and Sashihara, 2010). In private households, separation of egg yolk and egg white is usually performed manually, using the eggshells to transfer the egg yolk from one egg half to the other until all the egg white has run off the edge and into a recipient. This way, contact between the egg contents and the shells can easily occur. Therefore, as a proof of concept, the aim of this study was to investigate to which extent *Campylobacter* spp., present on the eggshell, may cross-contaminate the egg content when eggshells are used to separate egg yolk from egg white. In addition, *Campylobacter* spp. in food can change into a state where they are viable but nonculturable (VBNC) but able to keep their infection potential (Lv et al., 2020). Conventional culture methods are usually applied to culturable cells, but unsuitable for cells in VBNC status, while conventional qPCR can also detect DNA from dead cells (Lv et al., 2020). Therefore, another aim was to develop a method to detect viable (culturable and VBNC) cells of *C. jejuni* on eggshells, by using propidium monoazide in combination with qPCR (PMA-qPCR). Additionally, the developed PMA-qPCR method was verified by applying it to quantify viable cells of *C. jejuni* on shells of eggs that were purchased from retail.

Material and Methods

Egg samples. Specific Pathogen-Free (SPF) eggs (n = 27) were used to develop DNA extraction, qPCR, and PMA-qPCR methods, and for the cross-contamination analysis. SPF laying hens were raised for laboratory purposes under stringent biosecurity conditions and a disease monitoring system to ensure that the animals and their products are free from certain pathogens, including *Campylobacter* spp. SPF eggs were provided by the Clinic for Birds, Small Mammals, Reptiles, and Ornamental Fish from the LMU Munich, Germany, and were stored at 5.0 ± 0.5°C until used within 7 days.

To exemplarily investigate the occurrence of total and viable cells of *C. jejuni* on eggshells, using the developed PMA-qPCR method, 30 egg packs with 10 eggs per pack, purchased from different supermarkets in the Munich area (Germany), were used. Producers and batch numbers of egg packs were all different. The production systems were in barns (n = 10), free-range (n = 10), and organic farming (n = 10). After purchase, the eggs were stored at 5.0 ± 0.5°C and analyzed within 7 days. Corresponding to the EU Commission Regulation (EC) No 589/2008, the eggs were not washed or disinfected prior to selling at retail in Germany.

Culture of *C. jejuni* strain and preparation of standard dilution for qPCR. A reference strain of *C. jejuni* (DSM 4688) was subcultured on modified charcoal cefoperazone deoxycholate agar (mCCDA, VWR, Germany). The plates were placed in an anaerobic box containing Anaerocult™ C (Merck KGaA, Germany) to generate a microaerophilic atmosphere and incubated at 41.5°C for 48 h. After incubation, 3–4 colonies were taken off the plates using a sterile inoculating loop (Zefa, Germany) and mixed with 3 ml of 0.85% NaCl (Merck, Germany). The cell suspension was adjusted to a 1.0 McFarland turbidity (Densimat Densitometer, Biomérieux, France), corresponding to about 3.0×10^8 cfu/ml (Seeley et al., 1991). These freshly prepared *C. jejuni* suspensions were used to produce three inoculum types: the first one was to establish the standard dilution for qPCR, the second to artificially contaminate SPF eggs for cross-contamination analysis and the third to develop the viable/dead cells analysis by PMA-qPCR.

To establish the standard dilutions for qPCR, a fresh *C. jejuni* culture, adjusted to 1.0 McFarland turbidity standard, was serially diluted in 0.85% NaCl (1:10) up to the dilution 10^{-8}. A total of 100 µl from the last four dilutions (10^{-5}–10^{-8}) was spread on mCCDA and microaerophilically incubated at 41.5°C for 48 h. In the meantime, the original suspension was stored at −20°C to prevent further bacterial growth. After plate counting, the number of cells in the original suspension was adjusted to 10^7–10^2 cfu/ml with NaCl. In between, SPF egg was cracked, and egg white, egg yolk, and eggshell were separated. The eggshell was weighed and mixed with a dilution suspension containing 0.85% NaCl and 0.1% peptone (Merck, Germany) in a ratio 1:10 g/ml for 30 s by using a blender (Krups, Germany). Then, the suspension was filtered using Stomacher® bags with a lateral filter (VWR International, Germany) and 10 ml were filled in a falcon tube.

Subsequently, 900 µl of each egg component (egg white, egg yolk, and eggshell suspension) was mixed with 100 µl of inoculum (10^7–10^2 cfu/ml, as described in the previous paragraph), resulting in final concentrations of 10^6–10^1 cfu/ml egg white or egg yolk, and 10^7–10^2 cfu/g eggshell. The described procedure was performed in three biological replicates and three technical replicates for both SPF eggs and *C. jejuni* cultures. Each replicate was subjected to DNA extraction and qPCR analysis to determine the quantification cycle (C_q) values.

DNA extraction, qPCR, and sequencing. Eggshell, egg white, and egg yolk are very different in their composition. While the egg white consists of 87% water, 11% protein, and less than 1% fat, egg yolk has a water content of 50%, 11% protein and, with 32%, a high fat proportion (Sugino et al., 1996). Fat and protein act as PCR inhibitors and need to be eliminated during DNA extraction (Bernardo et al., 2007). Therefore, the DNA extraction method was adapted to the characteristics of each egg component.

The DNeasy Blood and Tissue Kit (Qiagen, Netherlands) was used to extract DNA from all sample types. When extracting DNA from the eggshell and inoculating suspension, 1 ml of the suspension was centrifuged for 1 min at 12,000 × *g*, 800 µl of supernatant was discarded and the remaining 200 µl were used for DNA extraction. For each egg yolk or egg white sample, 200 µl was directly proceeded to DNA extraction. A total of 200 µl of the sample was thoroughly mixed with 200 µl of ATL buffer by vortexing. Then, 40 µl of pro-

teinase K was added and mixed, and the reaction tube was incubated at 56°C for 10 min in a Thermomixer (Eppendorf, Germany). After adding 200 µl of AL buffer and vortexing, the mixture was further incubated at 56°C for 10 min. Then, 200 µl of absolute ethanol was added to the inoculating suspension, the eggshell suspension, and the egg white samples, while the egg yolk was mixed with 600 µl of solution containing 2-propanol (Merck, Germany) and hexane (Th. Geyer, Germany) in a 1:2 ratio. The following steps were performed following the manufacturers' instructions.

The decision of using 2-propanol–hexane mixture instead of ethanol for egg yolk was based on a comparative test of both solutions. For this, egg white and egg yolk were artificially contaminated with *C. jejuni* (10^6–10^1 cfu/ml in three biological replicates) and prepared into two subsamples for DNA extraction as described in the previous paragraph. While ethanol was applied to the first subsample, the second subsample was mixed with 2-propanol and hexane mixture. The C_q values obtained from both subsamples were compared, and the efficiency of the DNA extraction method was evaluated.

The qPCR with SYBR-Green I and primer pair pg50 (5´-ATGG GATTTCGTATTAAC-´3) and pg3 (5´-GAACTTGAACCGATTTG-´3; Oyofo et al., 1992) was applied to quantify *C. jejuni* in the eggshell suspension, egg white, and egg yolk. This primer pair can additionally detect *C. coli* (Oyofo et al., 1992). The fragment size of PCR products is 458 bp. The mixture for the qPCR contained 0.2 µM of each primer, 10 µl of SensiFAST™ SYBR No-ROX Kit (Bioline, United Kingdom), 2 µl of DNA and was filled up with water to a final volume of 20 µl. All qPCR runs were carried out using a Biorad CFX96 Touch™ thermocycler (Biorad, United States), and the following program was used: initial denaturation at 95°C for 3 min, followed by 50 cycles of denaturation at 95°C for 10 s, annealing at 50°C for 5 s, and elongation at 72°C for 15 s. The melting curve was subsequently analyzed by heating up the mixture from 65°C to 95°C in steps of 0.5°C for 5 s each.

The PCR products of artificially contaminated samples with C_q-values >38 and all positive egg samples from retail were purified using E.Z.N.A.® Cycle Pure Kit (VWR, Austria) and then sequenced (Microsynth, Austria). The individual sequences were aligned with the sequences available at GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi, NCBI, the United States) to confirm the targeted DNA as *C. jejuni*.

Determination of cross-contamination from eggshell to egg content by manual separation of egg components using eggshells. The manual separation of the egg components by persons in this study was performed in alignment to the technique commonly used in households: egg white and egg yolk were separated by cracking the egg on a flat surface with gloved hands and passing the egg yolk back and forth between the two halves of the eggshell, allowing the egg white to drip down into a petri dish. The latter step was repeated until just the egg yolk was left in the shell.

Four biological replicates were performed to determine the potential of cross-contamination. For the first three replicates, fresh *C. jejuni* culture was suspended in 0.85% NaCl, adjusted to 1.0 McFarland turbidity, and diluted to 10^{-2} and 10^{-3} (200 µl each). Both dilutions were used as inoculating suspensions. Per concentration, two SPF eggs were artificially contaminated by immersion into the inoculum for 2 min and were then left to dry in sterile metal egg cups for 10 min at room temperature. The dry egg was subsequently cracked, and the egg white was manually separated from egg yolk using eggshell. Altogether, 12 SPF eggs were inoculated in the first three replicates and the subsequent separation of egg components was always performed by the same person (person A).

For the 4th biological replicate, 10 SPF eggs were immersed in 200 µl of a freshly prepared *C. jejuni*-inoculum in the dilution 10^{-3} of a 1.0 McFarland turbidity suspension. Further five eggs were only immersed in 0.85% NaCl and used as negative controls. Separation of egg white and egg yolk was carried out by five independent persons (persons B to F). Each person separated three eggs, two being inoculated with *C. jejuni* and one negative control.

Once separated, the egg yolk was set into a separate sterile petri dish. Egg white as well as egg yolk were thoroughly mixed by stirring, using a 1-ml pipette tip for 2 min. The eggshell suspension was prepared (see section preparation of standard dilution for qPCR). Subsequently, 0.1 ml of each sample (egg white, egg yolk, and eggshell homogenate) was spread on mCCDA plates in three technical replicates and incubated at 41.5°C for 48 h under microaerophilic atmosphere. After colony counting, cfu per gram eggshell or per ml egg white or egg yolk were calculated. On the same day, that separation of egg content was performed, all egg components and inoculating suspensions were subjected to DNA extraction. For the subsequent qPCR, all replicates with the same *C. jejuni* concentration and matrix (see Table 1) were pooled and used as standards for the quantification of *C. jejuni* in each egg component.

Differentiation of viable and dead *C. jejuni* on eggshells. To differentiate viable from dead *C. jejuni*-cells on eggshells, a PMA-qPCR method was developed and applied to retail eggs. *C. jejuni*-culture (on mCCDA, incubated at 41.5°C for 48 h) was adjusted to 1.0 McFarland turbidity and serially diluted in SPF-eggshell suspensions up to the dilution 10^{-5}. One milliliter of freshly prepared *C. jejuni*-culture dilutions 10^{-3}, 10^{-4}, and 10^{-5} cfu/ml was filled in five reaction tubes: one untreated suspension; two treated with PMA (Biotium, United States) at a final concentration of 10 µM and 25 µM, respectively; and two heated at 72°C for 15 min to inactivate the viable cells and subsequently treated with 10 µM and 25 µM PMA, respectively. All five aliquots were incubated at 37°C with shaking at 550 rpm for 15 min in a Thermomixer (Eppendorf, Germany). Subsequently, the reaction tubes were put on ice and horizontally exposed to a halogen lamp (1,000 Watts, 30 cm distance) for 2.5 min per side and a total of 5 min. After that, the samples were centrifuged for 5 min at 15,000 × *g*, 980 µl supernatant was discarded, filled up with 0.85% NaCl to a final volume of 200 µl, and subjected to DNA extraction and qPCR. The described procedure was performed in three biological replicates.

Afterward, an appropriate final concentration of PMA was selected and applied to quantify viable *Campylobacter* spp. on the shells of retail eggs (n = 30 packs). From each pack, three eggs were cracked, the content was discarded, and the eggshells were pooled and weighed. Subsequently, an eggshell suspension was prepared (see section preparation of standard dilution for qPCR), and three 1-ml aliquots were sampled: one untreated suspension and two aliquots (fresh and heat-treated suspension) treated with 10 µM PMA. All three samples were then subjected to DNA extraction and qPCR.

Statistical analysis. The one-way analysis of variance (one-way ANOVA, Excel 2016) was used to analyze the statistical difference between the data sets obtained from the use of PMA (concentration 10 µM vs. 25 µM PMA vs. nontreated sample). The difference was considered statistically significant if p value < 0.05. Additionally, the

Table 1
qPCR C_q-values of artificially contaminated eggshell suspension, egg white, and egg yolk

Concentration of *C. jejuni* (cfu/ml)	C_q-values (at RFU = 500)		
	Eggshell	Egg white	Egg yolk
10^6	25.5 (±0.5)	–	–
10^5	29.0 (±0.5)	31.7 (±2.2)	37.5 (±1.1)
10^4	32.8 (±0.4)	35.6 (±1.3)	41.8 (±2.1)
10^3	36.4 (±1.0)	39.5 (±1.2)	44.0 (±0.7)
10^2	39.4 (±1.4)	44.0 (±1.2)	n.d.
10^1	43.8 (±1.8)	n.d.	n.d.

– = not determined; n.d. = not detected; RFU = relative fluorescence unit.

Pearson correlation coefficient (r, Microsoft Excel 2016) was used to determine the correlation between the number of *C. jejuni* in inoculating suspensions and on shells from inoculated eggs, as well as between the number of *C. jejuni* on spiked eggshells and egg whites. The strength of the correlation was interpreted as follows: r = 0–0.19 was regarded as very weak, 0.20–0.39 as weak, 0.40–0.59 as moderate, 0.60–0.79 as strong, and 0.8–1.0 as a very strong correlation (Evans, 1996).

Results and discussion

DNA extraction, qPCR, and sequencing. The matrix egg yolk is known to have a negative influence on the PCR amplification (He et al., 2007). Due to the high fat content, ethanol was substituted for a 2-propanol and hexane mixture to optimize the limit of detection. While 2-propanol is a polar solvent used to denature proteins present in the egg yolk and extract the polar lipids, hexane is especially useful for extracting neutral lipids (triglycerides). A mixing ratio of 1:2 is recommended, since high proportions of 2-propanol would increase the polarity of the solvent and triglycerides may therefore not be optimally extracted (Kovalcuks and Duma, 2014). By mixing the egg yolk suspension with this mixture, the detection limit for *C. jejuni* in egg yolk was optimized from 10^5 to 10^3 cfu/ml, which confirms the negative effect of the high lipid content in egg yolks for the exploitation of DNA. No positive effect of this mixture was observed for analyzing egg whites.

Table 1 shows the C_q-values of the three replicates after the artificial contamination of the egg components (eggshell, egg white, and egg yolk). The melting curve analysis was performed to ensure a similar melting temperature (81°C) for all positive samples. The detection limit was 10^1 cfu/ml for the eggshell suspension (corresponding to 10^2 cfu/g eggshell) and 10^2 cfu/ml for the egg white. Despite the optimized DNA extraction, only a $\geq 10^3$ cfu/ml *Campylobacter* spp. contamination in egg yolk could be detected.

High C_q-values generally indicate a very low target DNA concentration per sample. In case of insufficient specificity of the qPCR, high C_q-values may result from nonspecific PCR products or DNA from nontarget microorganisms. To rule out the latter assumptions, DNA products of the artificially contaminated eggshell samples with >38 C_q-values (containing *C. jejuni* 10^2 and 10^1 cfu/ml, see Table 1) were purified and sequenced. An identity of 99.3–100% to the *C. jejuni* strain DSM 4688 sequence (NCBI, accession no. CP019838.1), which is the same strain used for inoculating in this study, confirms the specificity and sensitivity of the applied qPCR to detect low numbers of *C. jejuni*.

Determination of cross-contamination of *C. jejuni* from eggshell to egg content. Table 2 shows the results of the analysis of the contaminated 22 SPF eggs by Persons A to F. The negative controls remained negative for *C. jejuni*. For the separation of egg white and egg yolk by Person A (1st–3rd biological replicates, n = 12), the qPCR results were compared with those of the culture method. For Persons B to F (4th replicate, n = 10), the culture method was not determined since the results from replicate 1st–3rd indicated sufficient results of using qPCR to detect *C. jejuni* in egg components, and to determine cross-contamination of these bacteria from eggshell to egg content.

The *C. jejuni* concentration in inoculating suspensions determined by qPCR ranged from 4.0 to 7.1 cfu/ml. While a *C. jejuni* contamination could be detected on all eggshell samples by qPCR, three culture samples were negative. Determined using qPCR, a cross-contamination from eggshell (1.9–4.0 $\log_{10}$ cfu/g) to egg white samples during manual separation could be detected in 68% of the tests (n = 15/22, 1.5–3.8 $\log_{10}$), but only in 14% (n = 3/22, PCR positive) of egg yolk samples.

The qPCR results from this study show that *C. jejuni* contamination levels in inoculated eggshells moderately correlate (r = 0.45) with *C. jejuni* concentrations in inoculation solutions and between that from the egg white (r = 0.53) with that found on eggshells. Regarding this result, each eggshell may have a variable capacity to carry *C. jejuni* on its surface (e.g., due to surface characteristics, such as roughness). A small variability in the manual separation technique may be expected, even if the procedure is performed by the same person. Therefore, the cross-contamination level from eggshell to egg white or yolk can vary, despite similar contamination levels on the eggshell.

In general, a greater number of artificially contaminated eggs may be desirable to assure the repeatability of the test, such as for cross-

Table 2
Cross-contamination of *C. jejuni* from the eggshell to the egg white and yolk after their manual separation using eggshell, determined by qPCR and culture method

Biological replicates	Person	Inoculum ($\log_{10}$ cfu/ml)	Egg No.	Eggshell ($\log_{10}$ cfu/g)		Egg white ($\log_{10}$ cfu/ml)		Egg yolk ($\log_{10}$ cfu/ml)	
				qPCR	culture	qPCR	culture	qPCR	culture
1	A	4.8	1	2.7	3.5	n.d.	n.d.	n.d.	n.d.
			2	2.6	3.1	n.d.	n.d.	n.d.	n.d.
		3.6	3	2.2	2.4	n.d.	n.d.	n.d.	n.d.
			4	1.4	2.6	n.d.	n.d.	n.d.	n.d.
2	A	6.6	5	3.4	3.2	2.4	2.3	n.d.	n.d.
			6	3.3	3.3	1.0	2.4	n.d.	n.d.
		5.5	7	3.1	2.0	n.d.	1.9	pos*	n.d.
			8	1.9	n.d.	1.7	1.7	pos*	n.d.
3	A	7.1	9	4.0	2.9	3.8	2.0	pos*	n.d.
			10	3.8	2.6	3.6	1.4	n.d.	n.d.
		5.8	11	3.4	n.d.	2.1	n.d.	n.d.	n.d.
			12	3.2	n.d.	2.2	1.0	n.d.	n.d.
4	B	4.0	13	3.3	–	2.0	–	n.d.	–
			14	2.9	–	2.4	–	n.d.	–
	C		15	3.3	–	1.5	–	n.d.	–
			16	3.2	–	1.8	–	n.d.	–
	D		17	3.2	–	2.3	–	n.d.	–
			18	3.2	–	2.3	–	n.d.	–
	E		19	3.6	–	n.d.	–	n.d.	–
			20	3.3	–	n.d.	–	n.d.	–
	F		21	3.2	–	2.2	–	n.d.	–
			22	3.2	–	2.0	–	n.d.	–

– = not determined; n.d. = not detected; * positive = a signal could be detected. but did not reach the threshold value.

contamination rates or loads of *C. jejuni* found in egg components. However, the primary goal of this study was to prove one variable: whether cross-contamination between eggshell and egg component can generally occur, in the sense of a proof of concept. The number of artificially contaminated eggs ($n = 22$) in the present study is in line with that addressed by Hair et al. (2018), who suggest sample-to-variable ratios of 15:1 or 20:1 (Memon et al., 2020; Rahman, 2023). With the result that out of 22 artificially contaminated eggs whose contents were separated using eggshells by six people, *C. jejuni* was found again, either by qPCR or culture, in the components of 16 eggs (73%), it was proven that cross-contamination can happen.

Suggested by some health authorities, eggshells should not be used to separate the egg white from the egg yolk, since *Salmonella* spp. and other pathogens on the surface of the eggshell can cross-contaminate the contents of the egg (NSW Food Authority, 2020; Queensland Health, 2023). The present study shows for the first time that manual separation of egg components might more likely result in cross-contamination of *C. jejuni* from the shell to the egg white than to the egg yolk. It is however to be considered that *C. jejuni* contamination below 10^3 cfu/ml may not be detected by qPCR due to the limitation of the DNA extraction method. On the other hand, in none of these PCR-positive egg yolk samples, *Campylobacter* spp. could be detected by culture.

The number of *C. jejuni* detected in positive egg whites was close to the limit of detection (10^2 cfu/ml). Although the load of *C. jejuni* per milliliter might seem low, 100 ml of contaminated egg white samples would contain a total of 10^4 viable cells, which might result in infection if used in not properly heated food products. As reported in some studies, the consumption of food contaminated with only a small amount of 500–800 viable cells of *C. jejuni* is sufficient to cause campylobacteriosis (Black et al., 1988; FSAI, 2011; RKI, 2018).

Differentiation of viable and dead *C. jejuni* on eggshells. Culture-based methods are generally viewed as the gold standard method to detect or to quantify a specific pathogen. However, isolation of *C. jejuni* is considered to be difficult, especially if in viable but non-culturable (VBNC) form (Kim et al., 2019). This is why the use of PMA was established in previous studies for the detection of viable *C. jejuni*, especially in chicken meat, since it is identified as an important source of campylobacteriosis in humans (EFSA, 2022). The applied PMA concentration varied among studies, e.g., 10 μg/ml (corresponding to 23.8 μM) (Josefsen et al., 2010), 20 μM (Lv et al., 2020), and 47–50 μM (Seinige et al., 2014) for chicken meat. In addition, the efficiency of PMA may be influenced by the sensitivity of the cell membrane of each bacterial strain (Fittipaldi et al., 2012), the size of the target amplicon, the sample type, or the light source (Pacholewicz et al., 2013).

In the present study, two PMA concentrations (10 and 25 μM) were tested with three different concentrations of viable (fresh culture) and dead *C. jejuni*. Summarized from three biological replicates, Figure 1 shows the comparison between eggshell suspensions containing untreated fresh cultures, and fresh cultures treated with 10 μM and 25 μM PMA, respectively. As no PCR amplification was observed from samples containing dead *C. jejuni* cells treated with PMA, data are not shown in the figure. In fresh cultures treated with 25 μM PMA, the number of detected viable *C. jejuni* cells was lower than in samples treated with 10 μM and untreated fresh cultures. However, the differences of all three variations (untreated vs. 10 μM vs. 25 μM PMA) were not statistically significant ($p > 0.05$), which demonstrates 10 μM PMA is sufficient to inhibit PCR amplification in samples containing <5.0 $\log_{10}$ dead cells/g eggshell. Therefore, this concentration was used for subsequent investigations on the presence of viable *C. jejuni* on 90 eggshells from 30 egg packs obtained from retail in Munich (Germany).

Regarding retail egg samples, contamination with *C. jejuni* could be detected in 23% ($n = 7/30$) of all packs, ranging from detectable (<2.0) up to 4.4 $\log_{10}$ cells/g eggshell (Fig. 2). Sequences of all positive eggshell samples showed a similarity of 99.5% to 100% to the *C. jejuni* sequences provided in Genbank (NCBI, accession no. AP026011.1, CP012250.1, CP040016.1, CP071585.1, KM396357.1, and MW713329.1). One pack from organic farming, two packs from free-range, and three packs from barn-raising systems were naturally contaminated only with dead *C. jejuni*. One egg pack obtained from the latter raising system was found to be contaminated with viable *C. jejuni* (3.3 $\log_{10}$ cfu/g) determined by PMA-qPCR. According to this, 3% (1/30) of the investigated egg packs contained viable *C. jejuni*, compared to the studies conducted in the same region (Germany), where the prevalence of culturable *Campylobacter* spp. in egg samples was between 0.4% and 0.5%. (Messelhäusser et al., 2011; Schwaiger et al., 2008). The detected viable *C. jejuni* were not further differentiated as to whether they were in culturable or VBNC status, since both statuses are potentially harmful for human health. As a prevalence study requires a higher number of investigated egg packs, it has to be mentioned that this study did not aim to determine the prevalence of viable *C. jejuni* on eggs sold at retail but rather to apply the developed PMA-qPCR to the field egg samples. The sequencing analysis of the PCR-positive samples was subsequently performed to assure the validity of the primer pair and qPCR protocol when applied to the field egg samples, since these eggs might be contaminated with a greater variety of bacteria compared to the artificially contaminated SPF.

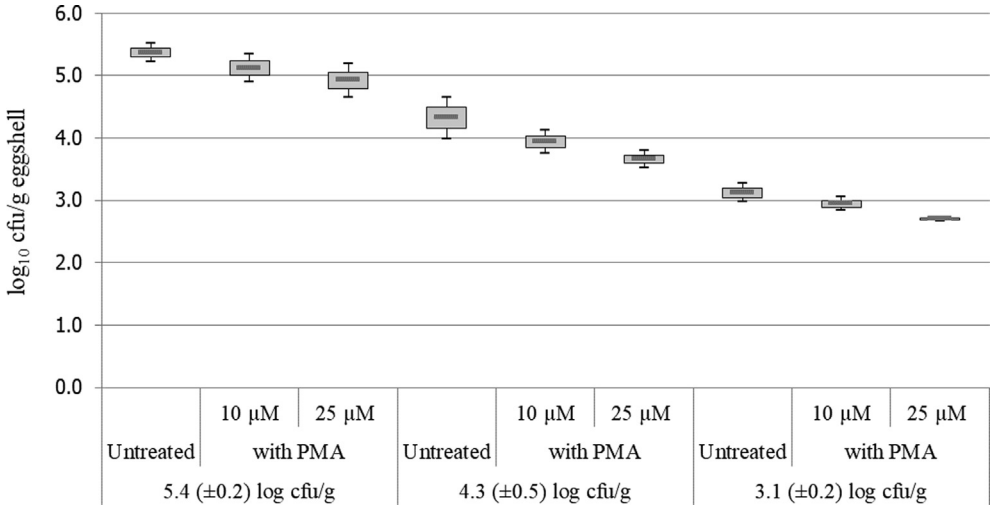

Figure 1. Number of total (untreated samples) and viable (PMA-treated) cells of freshly prepared *C. jejuni* in contaminated eggshell-suspensions.

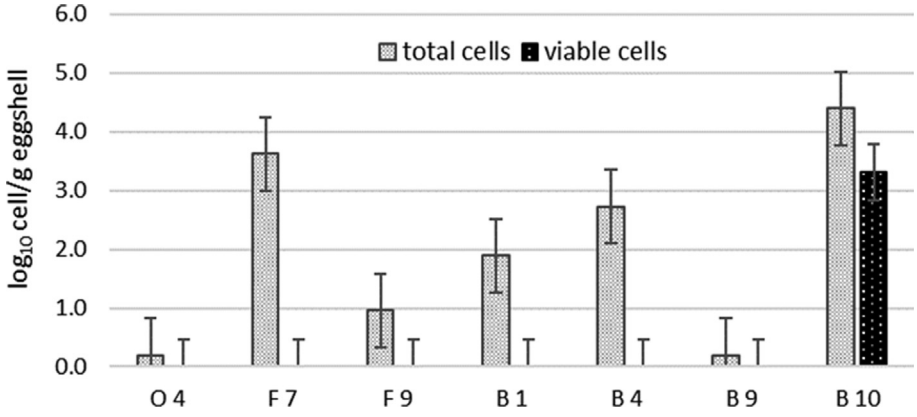

Figure 2. Retail egg samples (and their internal identification number) tested positive for total and viable cells (with error bars) of *C. jejuni*, determined by qPCR, and PMA-qPCR, respectively. O = organic farming, F = free range and B = barn raising system, n = 10 egg packs for each raising system.

In this study, both aims, the proof of cross-contamination from the eggshells to the egg content during manual separation of the egg components, and the establishment of a PMA-qPCR method to differentiate viable from dead *Campylobacter* spp. cells were achieved. Looking at current studies, eggs and egg products do not seem to be a relevant source of contamination regarding campylobacteriosis, since they are continuously responsible for less than 2% of all outbreaks (Batz et al., 2012; Greig and Ravel, 2009; Pires et al., 2010). In single cases, when egg contents of contaminated eggs are manually separated using eggshells and then processed for insufficiently heated food, illness caused by campylobacteriosis cannot be excluded. In addition, although the microorganism of choice was *C. jejuni,* other pathogens such as *Escherichia coli*, *Salmonella* spp., *Enterococcus* spp., *Pseudomonas* spp., *Staphylococcus* spp., and *Bacillus* spp. may also contaminate eggshells (Chaemsanit et al., 2015; Islam et al., 2018; Pesavento et al., 2017; Schwaiger et al., 2008). Therefore, proper handling of whole eggs and the remaining eggshells, in both professional and household kitchens, should be focused on to minimize the risk of a potential cross-contamination of different pathogens from eggshells to egg contents. Particularly, special care should be taken when processing food containing raw egg or not thoroughly heated products, where *Campylobacter spp.* or other pathogens are not completely inactivated before consumption.

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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