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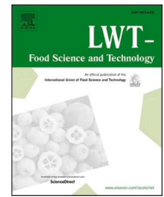

Using peptide nucleic acid fluorescence *in situ* hybridization (PNA-FISH) to detect *Campylobacter* spp. in food samples

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ABSTRACT

Foodborne diseases have a considerable negative impact on socioeconomic development globally and are an important cause of morbidity and mortality. Among the foodborne bacterial pathogens, *Campylobacter* spp. is recognized as the leading cause of foodborne illness. Fluorescent *in situ* hybridization using nucleic acid mimics (NAM-FISH) as probes, in particular peptide nucleic acid (PNA) probes, is a molecular technique that has emerged as an essential and resourceful tool for bacterial detection.

Here, we applied a PNA-FISH methodology, including pre-enrichment culture, for the specific detection of *Campylobacter* spp. in food matrices, more specifically fresh raw broiler meat and fresh raw pork. New PNA probes, including a blocker probe, have been designed for a 23S rRNA sequence. The PNA-FISH technique presented sensitivity and specificity values of 92.0% and 96.9%, respectively. In food matrcies, the best detection condition was achieved with a pre-enrichment of 48 h in Bolton broth, allowing a detection limit of 1 CFU/25 g. Compared to the ISO 10272-1:2017 reference method, this methodology showed similar performance in food matrices. The present study revealed that the developed PNA-FISH method is a promising alternative for detecting *Campylobacter* spp. in food samples.

1. Introduction

Campylobacter spp. are commensal, microaerophilic, gram-negative bacteria, regularly associated with domesticated animals farmed for meat, such as cattle, sheep, swine, and poultry (Elmi et al., 2020; Man, 2011). Nevertheless, *Campylobacter* has been considered one of the most reported gastrointestinal bacterial pathogens in humans (Lassen et al., 2022; Newell et al., 2010; Thames & Sukumaran, 2020). According to the official data from EFSA (European Food Safety Authority, 2022) and ECDC (European Centre for Disease Prevention and Control), included in the European Union One Health 2022 Zoonoses Report, there were recorded 137 107 campylobacteriosis cases and 34 deaths, representing more than 61.3% of human zoonotic events. Of the reported cases that provided information on the species, 87.6% were caused by *C. jejuni*, 10.7% by *C. coli*, 0.26% by *C. fetus*, 0.17% by *C. upsaliensis* and 0.12% by *C. lari*. Food-producing animals are considered the main source of *Campylobacter* infections in developed countries, and the consumption of poultry products, beef and pork are considered the main cause of campylobacteriosis. It is estimated that poultry consumption, in particular broiler meat, is responsible for the majority of human *Campylobacter* infections, especially *C. jejuni*, while pork consumption is more associated with cases caused by *C. coli* (Authority, 2023; Korsak et al., 2015*)*. Since the notification is required in most European Member States, food surveillance and testing should be a top-priority practice for most retailers, food-related companies, or public health authorities in order to avoid the release and dissemination of contaminated products in the food market (Abd El-Hack et al., 2021; Thames & Sukumaran, 2020). Over the past decades, the means by which foods are tested suffered a large shift, going from complete dependency on bacteriological protocols to the integration of advanced molecular

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methods (Harrison et al., 2022; Zhang, 2013).

Traditionally, *Campylobacter* analysis of foods is based on the isolation of microorganisms by culture methods, mostly based on ISO 10272-1:2017 (standard method for the detection and enumeration of *Campylobacter* spp. in food) (ISO-10272-1, 2017), and confirmation by visual, biochemical, immunological, or genetic means, either before or after enrichment (Harrison et al., 2022; Soto-Beltra et al., 2022). However, the latest update of the methodology already includes final confirmation by polymerase chain reaction (PCR) (10272-1:2017, 2023). The PCR methodology is a widely used molecular technique in laboratories nowadays, allowing a reduction in the time needed to confirm the final result, however it still has some limitations, such as false positives results associated with the presence of DNA from dead target cells (Wolffs et al., 2005).

In addition, culture-based methods are normally labor-intensive, and time-consuming, taking 2–3 days for initial presumptive results and up to more than 1 week for confirming the specific pathogenic microorganisms. Failure to isolate a pathogen from a contaminated sample or an underestimation of pathogen counts might also result from culture-based techniques' inability to identify viable but non-cultivable (VBNC) bacteria (Li et al., 2014). Consequently, the advance in food microbiology has been focused on rapid, reliable, and specific culture-independent methods. In that sense, Peptide Nucleic Acid - Fluorescence *In Situ* Hybridization (PNA-FISH) is one of the most promising techniques available for pathogens detection (Almeida et al., 2013; Rocha et al., 2019; Sousa et al., 2019). PNA-FISH is based on complementary binding of a fluorescently labelled PNA probe to a specific 16S or 23S rRNA sequence of the target microorganism (Cerqueira et al., 2013; Emerson et al., 2017; Nacher-Vazquez et al., 2022). So far, only one PNA-FISH method for the detection of *Campylobacter* spp. was published, targeting the 16S rRNA (Lehtola et al., 2005). However, genomic sequence databases used for probe design are constantly being updated, and the quality and quantity of sequences available may positively affect the accuracy of new probes (Cerqueira et al., 2008).

Therefore, in this work, PNA-FISH probes were designed using updated 16S/23S rRNA sequence databases for the specific detection of a genus *Campylobacter*. In order to improve method specificity, the most promising probe was further tested for accuracy and the addition of blocking probe was assessed as an improvement (Rocha et al., 2019; Stender et al., 2001). To evaluated the application of this method in food samples, the enrichment step was optimized, and the performance of the PNA-FISH method was compared to ISO 10272:2017, according to AOAC Performance Tested Methods℠ program.

2. Materials and methods

2.1. Bacterial strains and culture maintenance

A total of 82 bacterial strains from both the genus *Campylobacter* and other related genera belonging to the Centre of Biological Engineering (CEB) of the University of Minho (PT), were included in this study (Tables S1 and S2). *Campylobacter* spp. strains were maintained in Columbia blood agar (CBA) plates (Oxoid CM0331, UK) supplemented with 5% (v/v) defibrinated horse blood (Oxoid SR0050) at 41.5 °C in a CO_2 incubator (HERAcell 150i, Thermo Electron Corporation, USA), set to 5% O_2, 10% CO_2, and 85% N_2, and streaked onto fresh plates every 48 h. *Helicobacter* spp. and *Arcobacter butzleri* strains were also maintained in CBA plates (Oxoid) supplemented with 5% (v/v) defibrinated horse blood (Oxoid) under the same culture conditions as the *Campylobacter* spp. strains but at 37 °C. *Salmonella* spp., *Escherichia coli*, *Listeria monocytogenes*, *Citrobacter freudii,* and *Klebsiella pneumoniae* strains were maintained into tryptic soy agar plates (TSA; Liofilchem, Italy) at 37 °C for 24 h and streaked onto fresh plates every 48 h. *Wolinella succinogenes* were maintained into CBA plates (Oxoid) supplemented with 5% (v/v) defibrinated horse blood (Oxoid) at 37 °C in anaerobic conditions (80% N_2, 10% CO_2 and 10% H_2) using AnaeroGen Compact™ gas generation kits (Oxoid) and streaked onto fresh plates every 48 h.

2.2. ISO reference method

The various tests performed required a comparison of the results with a reference method. For *Campylobacter* spp., ISO 10272:2017 is the reference method (ISO-10272-1, 2017) commonly used by food safety institutions and, therefore, it was used as a basis for comparison. For this, Bolton broth (BB; Oxoid, CM0983), prepared according to the manufacturer's instructions with Bolton Broth Selective Supplement (Oxoid, SR0183) and 5% (v/v) lysed horse blood (Oxoid, SR0048), was used as enrichment media. For the analysis, test samples were homogenized into BB, in a test portion/enrichment medium ratio of 1:10 (wt/v), using a stomacher (Eco Blender II, VWR pbi, Italy) for 30 s and then incubated in a microaerobic atmosphere at 37 °C for 4 h plus 44 h at 41.5 °C in a CO_2 incubator (HERAcell 150i, Thermo Electron Corporation, USA), set to 5% O_2, 10% CO_2, and 85% N_2.

Following the enrichment, the isolation of *Campylobacter* spp. was carried out by inoculating the bacteria into two selective solid media: Modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA, Oxoid, CM0739 with CCDA Selective Supplement, Oxoid, SR0155) and Preston agar (prepared with Nutrient Broth No. 2 (Oxoid, CM0067), Preston *Campylobacter* Selective Supplement (Oxoid, SR0117) and *Campylobacter* Growth Supplement (Oxoid, SR0232), 5% (v/v) defibrinated horse blood (Oxoid) and 15 g/L agar. For this, a loopful of enriched food suspensions had been plated on the two selective solid media and incubated at 41.5 °C under the same microaerophilic conditions used for sample enrichment. The plates were inspected 48 h later to distinguish the presence of presumptive *Campylobacter* spp. colonies based on the characteristics they develop in each selective solid medium. Lastly, a colony suspected to be *Campylobacter* spp. was taken from each selective solid medium and subculture on CBA at 41.5 °C for 48 h under the same microaerophilic conditions as mentioned above. After that, well isolated colonies were confirmed by microscopic examination (based on morphology); aerobic growth at 25 °C; and the presence of oxidase. The morphology evaluation was performed under a microscope, taking into account the morphological characteristics of *Campylobacter* bacteria. The study of aerobic growth at 25 °C was performed by plating a suspected colony on a CBA plate at 25 °C for 48 h in aerobic atmosphere. For the detection of oxidase, a well-isolated colony was streaked onto a filter paper of the BD BBL DrySlide Oxidase test kit (Becton, Dickinson and Company, USA). The presence of a mauve, violet or deep blue colour within 10 s indicated a positive reaction. If at least one colony has a small, curved bacilli morphology, no growth at 25 °C in the aerobic atmosphere and a positive result in the oxidase test, *Campylobacter* spp. was present in the initial food sample.

2.3. Microscopy visualization

A Nikon Eclipse 80i epifluorescence microscope (Japan) with a NikonDS-Fi1 camera (Izasa, Japan) and a filter sensitive to the Alexa Fluor 594 molecule attached to the PNA probe (excitation, 530–550 nm; barrier, 570 nm; emission long-pass filter, 591 nm) was used to perform microscopic visualization. The additional filters in the microscope were applied to confirm that the cells did not exhibit autofluorescence. A negative control was performed concurrently with each experiment, using the same methods as the positive controls but without the addition of any probes during the hybridization protocol. Each image was captured using NIS-Elements B.R. 3.2 (Izasa, Japan) software at a magnification of ×600.

2.4. PNA-FISH method development

2.4.1. PNA probes design

For probe design, the Primrose program (Ashelford et al., 2002) coupled with 16S and 23S rRNA databases of Ribosomal Database Project (RDP) (version 11.5; http://rdp.cme.msu.edu/) and ARB-Silva (https://www.arb-silva.de/) (Teixeira et al., 2021) was used to identify sequences that could potentially be used as probes to detect *Campylobacter*. The identified sequences were analysed using empirical formulas for predicting the thermal stability of PNA/DNA duplexes to determine the theoretical melting temperature, Gibbs free energy, GC percentage, and number of contiguous self-complementary nucleotides (Giesen et al., 1998; Nacher-Vazquez et al., 2022; SantaLucia et al., 1996). The theoretical specificity and sensitivity of the selected probe were calculated using the Probe Match (RDP) and Test Probe (ARB--SILVA) tools, as previous described by (Almeida et al., 2010; Nacher--Vazquez et al., 2022). Selected sequences were then synthesized (Panagene, Daejon, Korea) and the N-terminus of the oligonucleotide was attached to an AlexaFluor® 594 molecule, via a double 8-amino-3, 6-dioxaoctanoic acid (AEEA) linker.

2.4.2. Blocker probe design

To avoid the non-specific binding to non-*Campylobacter* species, namely *E. coli* and *Salmonella* spp., a blocker probe was designed. The EBI website's Clustal Omega program (https://www.ebi.ac.uk/Tools/msa/clustalo/) was used to conduct the alignments on the 23S rRNA sequences obtained from the ARB-SILVA database. The blocker probe was designed to hybridize specifically with a mismatch sequence of 3 nucleotides of *E. coli* and *Salmonella* strains. The melting temperature and free energy was evaluated to ensure that both blocker and detection probes have similar hybridization conditions (Giesen et al., 1998; Rocha et al., 2019; Yilmaz & Noguera, 2004). Probe synthesis was performed in the same way as the detection probe, but lacking any linker or fluorochrome attached.

2.4.3. Hybridization conditions optimization

To assess the optimal hybridization conditions, the protocols based on (Almeida et al., 2010; Cerqueira et al., 2020; Oliveira et al., 2021), were employed with some modifications. 20 μL of each bacterial sample were dispensed in 8 mm well slides (Marienfeld, Lauda-Königshofen, Germany). For comparison assays/enrichement optimization of *Campylobacter* spp., broth-enriched samples were used, while for specificity/sensitivity assays bacterial suspensions ($\sim 10^8$ cells/mL) in PBS from fresh plated cultures were used. After drying, smears were first fixed, and then covered with 20 μL of hybridization solution with 200 nM of the detection probe (Panagene, South Korea) or a mixture of the detection probe and the blocker probe (Panagene) (1:1). as described by (Almeida et al., 2013; Cerqueira et al., 2020). Then the samples was placed in moist chambers covered with coverslips, and incubated for approximately 60 min at different temperatures, ranging from 55 °C - 59 °C. After hybridization, a coupling jar containing prewarmed washing solution was used to remove the non-binding probe as described (Almeida et al., 2013; Cerqueira et al., 2020). Lastly, the samples were air-dried, mounted with a drop of non-fluorescent immersion oil, and covered with a coverslip. Based on the optimal hybridization conditions, the PNA-FISH protocol was established as described in this section.

2.4.4. Specificity and sensitivity assay

For the specificity and sensitivity assay, 50 *Campylobacter* strains and 32 related non-*Campylobacter* strains were used. All strains used as well as their source/origin are documented in Tables S1 and S2. A small loopful of biomass from 24 h cultures of each bacterial strain was suspended directly in 3 mL of BB. The cell suspensions were then incubated at 37 °C for 4 h plus 44 h at 41.5 °C in a CO_2 incubator (HERAcell 150i), set to 5% O_2, 10% CO_2, and 85% N_2. After incubation, 1 mL of enriched suspensions were centrifuged at 10 000 g for 5 min and the pellet was resuspended with 0.1% Tween-80 solution. The samples were then blind coded, and mixed, and 20 μL of each treated enriched solution was placed on a microscope slide and tested in duplicate according to the PNA-FISH procedure previously described. Finally, the experimental values of sensitivity and specificity of the PNA-FISH method were calculated using the Clinical Calculator 1 software, available in http://vassarstats.net/.

2.5. Application of PNA-FISH method in food samples

2.5.1. Optimization of the enrichment step

The enrichment step optimization was performed with two enrichment broths: Bolton broth (BB; Oxoid, CM0983) and Preston Broth (PB) prepared with Nutrient Broth No. 2 (Oxoid, CM0067), Preston *Campylobacter* Selective Supplement (Oxoid, SR0117) and *Campylobacter* Growth Supplement (Oxoid, SR0232). All contained 5% (v/v) lysed horse blood (Oxoid, SR0048) and were prepared according to the manufacturer's instructions. In order to assess real conditions of enrichment, fresh raw broiler meat and fresh raw ground pork samples were acquired from a local retailer (Braga, Portugal).

For the artificial contamination, 25 g samples of each matrix were directly inoculated in stomacher bags with filters (VWR, USA) containing the amount of cells of *C. jejuni* CNET 90 or *C. coli* CNET 20, ensuring three inoculation levels on the beginning of the experiment: 1 CFU/25 g, 10 CFU/25 g, and 100 CFU/25 g. Cell concentrations were confirmed by plating on CBA. One non-inoculated food sample was included in each experiment to secure primary *Campylobacter* absence. After inoculation, microorganisms were allowed to rest in the matrix at 4 °C in the refrigerator for 24 h. Then, the 25 g test portions were mixed with 225 mL of BB or PB, homogenized in a stomacher (Eco Blender II) for 30 s, and incubated at 37 °C for 4 h followed by 44 h at 41.5 °C in a CO_2 incubator (HERAcell 150i, Thermo Electron Corporation), set to 5% O_2, 10% CO_2, and 85% N_2. After each enrichment, 20 μL samples were placed directly on a microscope slide and the PNA-FISH procedure was performed as described above. Simultaneously, confirmation was performed by culture method as previously described in the ISO method (ISO-10272-1, 2017). Two independent assays with three replicates of each inoculation level were performed.

2.5.2. Reduction of autofluorescence signal

During the optimization of the enrichment step using PNA-FISH, it was observed a strong autofluorescence signal, hindering the results confirmation under the microscope. As such, the artificially inoculated food samples were homogenized in 225 mL of BB and incubated as described above; after that, some approaches were applied in order to try to reduce the autofluorescence. As some studies suggest that red blood cells may confer autofluorescence to samples analysed by FISH (Almeida et al., 2010), a BB enrichment without the addition of lysed horse blood was tested in an independent assay. Alternatively, some additional treatment steps were introduced before the PNA-FISH procedure, trying to remove potential autofluorescent food particles: (1) 15 μL of enriched suspensions were mixed with 15 μL of a 1% Triton X-100 (Sigma-Aldrich) solution directly on the microscope slides to emulsify the fatty compounds, as previously reported by (Almeida et al., 2013); (2) 1 mL of enriched suspensions was centrifuged at 900 g for 1 min to sediment food particles, as suggested by Stevens and Jaykus (2004) (Stevens & Jaykus, 2004); (3) 1 mL of enriched suspensions was diluted (1:2 dilution) in dH_2O to dilute autofluorescent food particles; (4) 1 mL enriched suspensions was centrifuged at 10 000 g for 5 min and the pellet was then resuspended with a 0.1% Tween-80 (Sigma-Aldrich) solution to emulsify the fat compounds and disrupt possible hydrophobic and electrostatic interactions between the target organism and the food particles, as suggested by Stevens and Jaykus (2004) (Stevens & Jaykus, 2004). Following enrichment, 20 μL of each enriched suspensions were placed on microscope slides and the PNA-FISH method was

performed as described above. Finally, the PNA-FISH results from the different treatments were compared with no-treated samples to select the technique that allowed the best reduction of autofluorescence without compromising the enrichment performance.

2.6. PNA-FISH performance based on AOAC performance tests

2.6.1. Food matrix comparison test

After finishing the optimization of the PNA-FISH method, a comparison with the reference method for *Campylobacter* detection, based on ISO 10272-1: 2017 (ISO-10272-1, 2017) was performed. In here, fresh raw broiler meat and fresh raw ground pork were tested as they are the two food matrices considered the main transmission vehicles of *Campylobacter* spp. All food matrices were obtained from a local retailer (Braga, Portugal) and stored at 4 °C ± 2 °C.

For each matrix, three bulk batches were prepared, one non-inoculated (NI) was used to verify the presence of *Campylobacter* sp., a low level (LL) (approx. 0.2–2 CFU/25 g); and high level (HL) (approx. 2–10 CFU/25 g). For this purpose, *C. jejuni* NCTC 11168 and *C. coli* NCTC 11366 inoculums were used to simulate the natural contamination of the broiler meat and pork meat samples, respectively, during the production and storage processes. The other portion served as the uncontaminated level (control).

After the artificial contamination, all food samples were well mixed by kneading, with extreme care to achieve as close as possible a homogeneous distribution of microorganisms and then, the microorganisms were allowed to equilibrate in the matrix at 4 °C ± 2 °C for 48 h, as required by the ISO 10272-1: 2017. After that, the LL inoculated samples were divided into 20 replicate test portions of 25 g and the HL inoculated samples were divided into 5 replicate test portions of 25 g and placed in sealed stomacher bags with filters. In addition, 5 replicates of the uncontaminated samples from each matrix were also placed in sealed stomacher bags. Table 1 shows the details of the matrices, contamination levels and replicates required for this type of test. After preparation of the test portions, the PNA-FISH method and the ISO method were performed as previously described. Finally, the data obtained were analysed by Probability of Detection (POD) statistics for each food matrix. The POD was determined by dividing the total number of trials by the number of successful outcomes (Wehling et al., 2011). The POD was calculated for the PNA-FISH method results, POD_C, the culture reference method results, POD_R, and the difference in the PNA-FISH method results and reference method results, $dPOD_C$.

2.6.2. Most probable number (MPN) analysis

The food matrix comparison test was combined with the Most Probable Number (MPN) approach to determine the actual number of organisms introduced in food samples. The equivalent bulk batch was prepared into samples of 50 (× 5 replicates), 25 (× 20 replicates) and 10 (× 5 replicates) each for low-level (LL) estimation. Additionally, samples of 25 (× 5 replicates), 10 (× 5 replicates) and 5 (× 5 replicates) g each were produced from the corresponding bulk batch for high level (HL) estimate. Each test portion was analysed with the culture method enrichment and by PNA-FISH procedure as previously described. Finally, the number of positives from the 3 MPN levels of each food matrix were used to determine the MPN values and the 95.0% confidence intervals using the MPN calculator available at http://www.lcfltd.com/customer/LCFMPNCalculator.exe.

Table 1
Food matrix comparison test design with the details for the matrices, target strains, contamination levels and replicates.

Group A: Meat Products			
Food Matrix	**Strain**	**Contamination Level**	**Number of Replicates**
Fresh raw broiler meat	*C. jejuni* NCTC 11168	0 CFU/25 g	5
		0.2–2.0 CFU/25 g	20
		2.0–10 CFU/25 g	5
Fresh raw ground pork	*C. coli* NCTC 11366	0 CFU/25 g	5
		0.2–2.0 CFU/25 g	20
		2.0–10 CFU/25 g	5

2.6.3. Ruggedness test

The ruggedness test measures how successfully the method can withstand minor changes in method parameters that can be anticipated to happen when the method is used by an end user. Three parameters were selected to vary in this test: hybridization time, hybridization temperature; and time to result after mounting. In addition, an experiment factorial design was used to obtain a more significative analysis of the selected parameters (Table 2).

For this purpose, fresh raw broiler meat was pre-screened for natural contamination as described above. Thereafter, the matrix was divided into 20 replicates of 25 g of test portion and placed directly into stomacher bags with filter. Of these, 10 test portions were artificially contaminated with approximately 1 CFU/25 g of *C. jejuni* NCTC 11168 and 10 test portions were artificially contaminated with 10 CFU/25 g of *E. coli* CECT 515. The samples inoculation was performed as previously described. Then, the contaminated test portions were homogenized and incubated with BB media. Following incubation, the PNA-FISH method was performed according to Table 2. Finally, probability of detection (POD) was calculated for each condition.

3. Results and discussion

3.1. PNA probes design

In silico analysis identified two potential sequences (one targeting 16S and the other 23S rRNA) able to detect *Campylobacter* spp., including all the *C. jejuni*, *C. coli* and *C. lari*. The 23S rRNA probe, however, presented a less number of nontarget sequences matches (1 human gut metagenome and 1 *Sulfurimonas autotrophica*) and for that reason was selected for further study. The PNA oligomer sequence obtained was N – TAGCAGTGTCAAGC – C (5′-3′). The theoretical specificity and sensitivity of the probe were further evaluated and the search confirmed that the detection probe had detected three non-*Campylobacter* sequences in a total of 110 (specificity of 97%) and detected 105 *Campylobacter* sequences out of 107 present in the databases (sensitivity of 98%).

The detection probe was then tested for several temperatures, ranging from 55 to 59 °C for assessing the best hybridization conditions. However, to increase the specificity of the method, a Blocker probe was also designed (N - ATGTTCAGT GTCAAG – C) to block non-specific binding to non-*Campylobacter* species, namely *E. coli* and *Salmonella* spp., since some cross-hybridization was detected in the preliminary experiments of hybridization temperature assessment (Table S3). These two bacteria are also foodborne pathogens and can pose a high risk of

Table 2
Factorial design of the test taking into account the selected parameters. For each parameter, a lower value and a higher value was tested (except for the hybridization time which was tested two lower values).

Treatment Combination	Hybridization time	Hybridization temperature	Time to result after mounting
1	30 ± 5 min	52 ± 1 °C	30 ± 5 min
2	30 ± 5 min	52 ± 1 °C	90 ± 5 min
3	30 ± 5 min	62 ± 1 °C	30 ± 5 min
4	30 ± 5 min	62 ± 1 °C	90 ± 5 min
5	45 ± 5 min	52 ± 1 °C	30 ± 5 min
6	45 ± 5 min	52 ± 1 °C	90 ± 5 min
7	45 ± 5 min	62 ± 1 °C	30 ± 5 min
8	45 ± 5 min	62 ± 1 °C	90 ± 5 min
9 (Baseline)	60 ± 5 min	57 ± 1 °C	0

unreliable diagnosis if one of these bacteria is present in the sample. The addition of this blocker probe (in a ratio of 1:1), have allowed the best hybridization conditions at 57 °C (Table S3), as it was observed an improved fluorescent signal compared to 59 °C on microscope visualization.

3.2. Specificity and sensitivity of the PNA-FISH method

To determine the specificity and sensitivity of these probes, experiments with PNA-FISH have been carried out after optimized hybridization conditions on 82 available strains. Since this method includes an enrichment step that influences the growth of all bacteria, this assay was performed using pure cultures from each species/strains grown in enrichment medium (BB) as described above.

Of the 50 *Campylobacter* strains tested, 46 were detected and 4 were not detected (*C. jejuni* CNET 110, *C. upsaliensis* DSM 5365, *C. sputorum* subsp. bubulus DSM 5363 and *C. mucosalis* DSM 21682, corresponding to a sensitivity rate of 92.0% (95.0% CI: 79.9%–97.4%) (Table S1).

The identity of these strains was subsequently confirmed as *Campylobacter*, using a latex agglutination test specific for the identification of enteropathogenic *Campylobacter* spp. (Oxoid). The negative results of these three strains may therefore be explained by the alternative growth requirements and which may not been met by the enrichment step followed. In the DSM collection database, it is indicated that *C. upsaliensis* DSM 5365 requires incubation in microaerophilia with H_2 (i.e., 5–6% O_2, 4–10% H_2, 4–10% CO_2, 75–87% N_2); *C. sputorum* subsp. *bubulus* DSM 5363 reveals difficulty in growing in liquid medium containing blood; and *C. mucosalis* DSM 21682 requires anaerobic incubation (Goossens et al., 1990; Moss et al., 1990). Regarding *C. jejuni* CNET 110, although doesn't have different growth requirements, it was suspected that its slower growth hinder the detection of this strain by PNA-FISH.

In turn, of the 30 non-*Campylobacter* strains tested, only one gave a positive result (*H. pamatensis* CIP 104249) (specificity of 96.9% (95.0% CI: 82.0%–99.8%)) (Table S2), confirmed by the latex agglutination test specific for the identification of enteropathogenic *Campylobacter* spp. (Oxoid). In fact, the latex agglutination test instructions state that its possible to obtain cross-reactivity with this particular species. This may be related to the phylogenetic proximity between the genera *Helicobacter* and *Campylobacter*. Both are part of the same order *Campylobacterales* and, therefore, there is a genetic proximity that may result in similar phenotypic characteristics and growth behaviours (Lastovica et al., 2014). Thus, we hypothesise that this specific *Helicobacter* strain is phylogenetically closer to the bacteria of the genus *Campylobacter* than the other bacteria of its genus, and therefore survive the selective effect of the enrichment step and result in the weak fluorescence signal observed. A more in-depth analysis would be needed to prove this hypothesis.

3.3. Enrichment step optimization

To adapt the PNA-FISH method to the detection of artificially contaminated food matrices two enrichment broths were initially tested: BB and PB, both currently recommended by ISO 10272-1:2017 (ISO-10272-1, 2017) and compared by culture method. As observed in Table 3, the samples enriched in BB resulted in a higher number of *Campylobacter* positive samples, detected by PNA-FISH, 91.70% (33/36), than in PB, where only 63.90% (23/36) of inoculated samples were *Campylobacter*-positive. More specifically, enrichment in BB resulted in a positive detection of 4–5 out of 6 samples from both food matrices inoculated with 1 CFU/25g of *C. jejuni*/*C. coli*, while enrichment in PB resulted in a positive detection of only 2 out of 6 samples. Enrichment in the BB also resulted in a positive detection of all samples from the other two levels of inoculation of both food matrices (10 CFU/25 g and 100 CFU/25 g), but the enrichment in the PB resulted only in a positive detection of all samples in the highest inoculation level (100 CFU/25 g). Furthermore, all results were concordant to the (ISO-10272-1, 2017) indicating that the PNA-FISH method has a similar sensitivity compared to the traditional culture method. Since this technology is in its infancy in what regards the food safety area, there's still a need for the method to be proved as a good alternative in the detection of these pathogens (Rohde et al., 2015). However with the results obtained in the present study, it is possible to demonstrate the predictability of PNA-FISH on the detection of food microorganisms.

Table 3
PNA-FISH and culture (ISO-10272-1, 2017) results for the detection of *C. jejuni* and *C. coli* in different food matrices after enrichment with BB and PB. Food samples were artificially inoculated and subjected to a refrigerated storage period of 24 h. The results presented comprise the two independent assays.

Contamination level (CFU/25 g) [a]	Bolton broth			
	C. jejuni CNET 90 Fresh raw broiler meat		*C. coli* CNET 20 Fresh raw ground pork	
	PNA-FISH	Culture	PNA-FISH	Culture
0	- (0/2)	- (0/2)	- (0/2)	- (0/2)
1	+ (4/6)	+ (4/6)	+ (5/6)	+ (5/6)
10	+(6/6)	+ (6/6)	+ (6/6)	+ (6/6)
100	+ (6/6)	+ (6/6)	+ (6/6)	+ (6/6)
Contamination level (CFU/25 g) [a]	**Preston broth**			
	C. jejuni CNET 90 Fresh raw broiler meat		*C. coli* CNET 20 Fresh raw ground pork	
	PNA-FISH	Culture	PNA-FISH	Culture
0	- (0/2)	- (0/2)	- (0/2)	- (0/2)
1	+ (2/6)	+ (2/6)	+ (2/6)	+ (2/6)
10	+ (3/6)	+ (3/6)	+ (4/6)	+ (4/6)
100	+ (6/6)	+ (6/6)	+ (6/6)	+ (6/6)

[a] Real concentration of bacteria (CFU/25 g): 1st assay – *C. jejuni*: 2.0 ± 0.6| 9.0 ± 2.0| 88 ± 3.1; *C. coli*: 2.0 ± 0.0| 31.0 ± 11.7| 168.0 ± 21.4; 2nd assay – *C. jejuni*: 1.3 ± 0.6| 9.0 ± 2.0| 90.0 ± 4.4; *C. coli*: 1.0 ± 0.0| 11.0 ± 3.0| 99.3 ± 3.2.

3.4. Reduction of autofluorescence signal

A strong background fluorescence was observed in some samples, which affected the visualization and confirmation of the PNA-FISH outcome. This standard enrichment in BB resulted in the positive detection of 16 samples of both food matrices inoculated with *C. jejuni* CNET 90 and *C. coli* CNET 20. However, a strong background fluorescence was perceived (Fig. 1) which in some cases made it difficult to confirm the result of PNA-FISH. Initially, it was thought that the background fluorescence could result from the autofluorescence of red blood cells as previously reported by (Almeida et al., 2010). Thus, an enrichment step using BB without addition of lysed horse blood was tested. However, this phenomenon is also often associated with particles of the food matrices, such as fatty compounds, which exhibit fluorescence in the observed spectrum range (Almeida et al., 2013). Therefore, four different pre-hybridization approaches were tested in the enriched samples. Nevertheless, more important than eliminating or reducing autofluorescence, it is essential to ensure the PNA-FISH previously stablished limit of detection (Table 4). The enrichment in BB without addition of lysed horse blood resulted in the positive detection of 10 samples from both food matrices, detecting only one sample of the lowest inoculation level of *C. jejuni* CNET 90. The 1:2 dilution in dH_2O of the enriched samples resulted in a positive detection of 10 samples from both food matrixes. These two protocols resulted in the higher number of negative results compared to standard enrichment with direct analysis by PNA-FISH. In addition, the samples analysed by these two techniques showed little or no decrease in autofluorescence (Fig. S1).

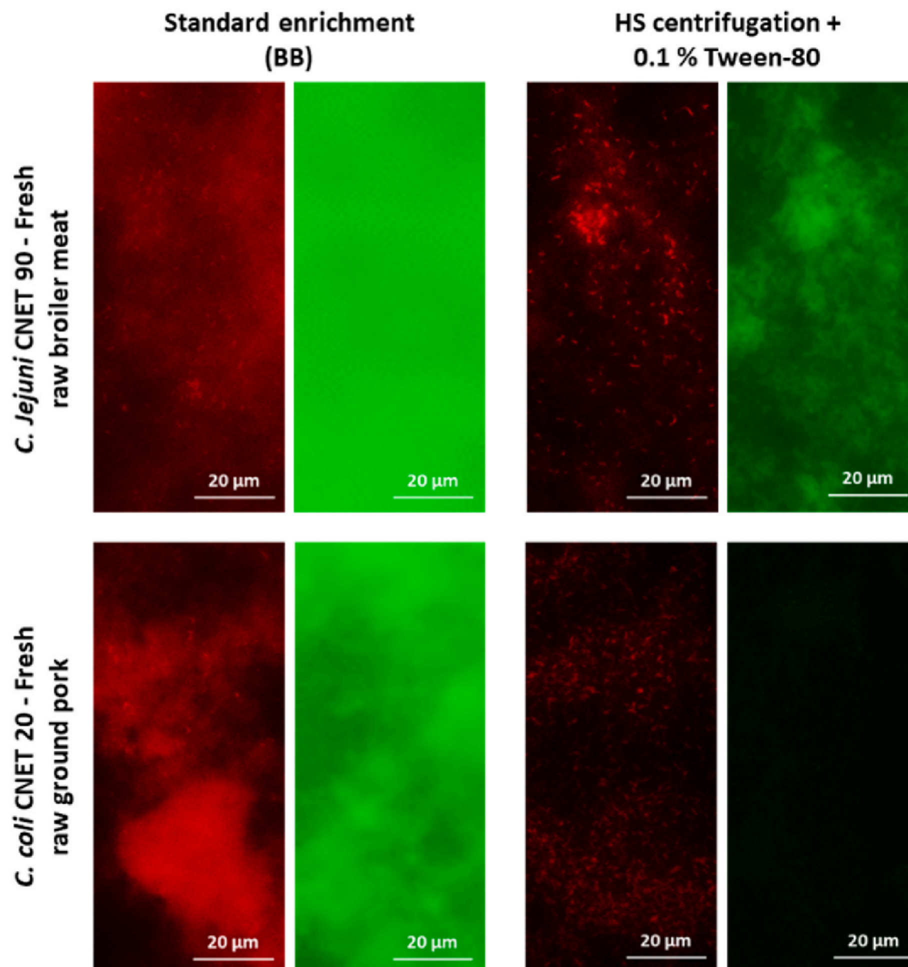

Fig. 1. PNA-FISH outcome for both food matrices artificially inoculated with 100 CFU/25 g of *C. jejuni* CNET 90 and *C. coli* CNET 20. Results were obtained using a PNA-FISH protocol without additional treatment of BB enriched samples and with additional high-speed centrifugation of the enriched samples with a 0.1% Tween-80 solution.

Table 4

PNA-FISH results for the detection of *C. jejuni* CNET 90 and *C. coli* CNET 90 in different food matrices after 48 h enrichment with BB. Results were obtained using a direct hybridization protocol after enrichment with BB (with and without addition of lysed horse blood) and using different autofluorescence reducing-steps before the PNA-FISH procedure. (+) Indicates condition.

***C. jejuni* CNET 90** **Fresh raw broiler meat**						
Contamination level (CFU/25 g) [a]	**Treatment**					
	BB (standard condition)	**BB without blood**	**1% Triton X-100**	**Low-speed centrifugation**	**1:2 Dilution**	**High-speed centrifugation + 0.1% Tween-80**
0	- (0/1)	- (0/1)	- (0/1)	- (0/1)	- (0/1)	- (0/1)
1	+ (2/3)	+ (1/3)	- (0/3)	+ (1/3)	- (0/3)	+ (1/3)
10	+ (3/3)	+ (1/3)	+ (1/3)	+ (3/3)	+ (2/3)	+ (3/3)
100	+ (3/3)	+ (3/3)	+ (3/3)	+ (3/3)	+ (3/3)	+ (3/3)
***C. coli* CNET 20** **Fresh raw ground pork**						
Contamination level (CFU/25 g) [a]	**Treatment**					
	BB (standard condition)	**BB without blood**	**1% Triton X-100**	**Low-speed centrifugation**	**1:2 Dilution**	**High-speed centrifugation + 0.1% Tween-80**
0	- (0/1)	- (0/1)	- (0/1)	- (0/1)	- (0/1)	- (0/1)
1	+ (2/3)	- (0/3)	+ (1/3)	+ (1/3)	+ (1/3)	+ (2/3)
10	+ (3/3)	+ (2/3)	+ (3/3)	+ (3/3)	+ (1/3)	+ (3/3)
100	+ (3/3)	+ (3/3)	+ (3/3)	+ (3/3)	+ (3/3)	+ (3/3)

[a] . Real concentrations of bacteria (CFU/25 g): 1st assay: *C. jejuni:* 2.3 ± 1.5| 31.0 ± 6.0| 112.3 ± 9.9; *C. coli:* 1.7 ± 1.5| 9.0 ± 2.6| 93.3 ± 4.7.

These results demonstrate that the addition of lysed horse blood to the enrichment broth is essential to ensure the desired level of detection (1 CFU/test portion) and the red blood cells were not responsible for the observed autofluorescence. Dilution of the samples also appears to dilute the number of *Campylobacter* cells to levels not detectable by the PNA-FISH method, reducing the desired limit of detection.

Treatment of samples enriched with 1% Triton X-100 directly on the slides resulted in a positive detection of 11 samples from both food matrices, also detecting only one sample of the lowest inoculation level of *C. coli* CNET 20. In turn, the low speed centrifugation of the enriched samples resulted in a positive detection of 14 enriched samples from both food matrices. Finally, high-speed centrifugation of the enriched samples followed by resuspension of the bacterial pellet with a 0.1% Tween-80 solution resulted in a positive detection of 15 samples from both food matrices. These three protocols have demonstrated a better performance in reducing the autofluorescence observed. However, the treatment of the enriched samples with Triton X-100 showed more negative results in comparison with the standard enrichment than the other two treatments. The explanation found for this was that the use of the detergent directly on the slides should interfere with the fixation step, leading to cells detachment/loss during washing procedures in the PNA-FISH method. In turn, low speed centrifugation resulted in two more negative results than the direct analysis of the enriched samples, whereas high speed centrifugation and pellet resuspension with a 0.1% Tween-80 solution resulted in only one more negative result, and was selected as an intermediate step (Fig. 1).

3.5. Food matrix comparison test

After the optimization of PNA-FISH method in the previous experiments, a validation was performed in two main food matrices associated with contamination: broiler meat and pork. A total of 30 matched sample replicates of each food matrix were evaluated by both detection methods as part of the method comparison study. Within the sample sets, there were 5 uninoculated samples (0 CFU/25 g), 20 low level inoculated samples (0.2–2 CFU/25 g), and 5 high level inoculated samples (2–10 CFU/25 g). For this purpose, *C. jejuni* NCTC 11168 and *C. coli* NCTC 11366 inoculums were prepared and used to artificially contaminate fresh raw broiler meat and fresh raw ground pork, respectively (Table 5).

For the low inoculation level, there were 12 out of 20 positive samples of fresh raw broiler meat and 11 positive samples of fresh raw ground pork, for both the PNA-FISH method and the culture reference method. Similarly, for the high inoculation level, there were 5 out of 5 positive samples for both matrices at both methods. These results demonstrate a similar performance between the two methods, proving that the PNA-FISH method guarantees the same level of detection as the standard culturing method. Overall, this method present similar performance to previously published PNA-FISH methods, namely a LmPNA1253 probe (Rocha et al., 2019) and the Probe4Cronobacter (Sousa et al., 2019), for the detection of *Listeria* and *Critrobacter* in food samples, respectively. These two PNA-FISH also being able to detect microorganisms at low and high levels of contamination similar with performance to the reference method (Rocha et al., 2019; Sousa et al., 2019).

Table 5
Comparative results for the detection of *C. jejuni* NCTC 11168 and *C. coli* NCTC 11366 in fresh raw broiler meat and fresh raw ground pork, respectively, by PNA-FISH and the ISO reference culture method (ISO-10272-1, 2017).

Fresh raw broiler meat *C. jejuni* NCTC 11168			**Fresh raw ground pork *C. coli* NCTC 11366**		
MPN determination (CFU/25 g)	**PNA-FISH**	**ISO 10272-1:2017**	**MPN determination (CFU/25 g)**	**PNA-FISH**	**ISO 10272-1:2017**
Control	0/5	0/5	Control	0/5	0/5
0.85 (0.51–1.40)	12/20	12/20	0.84 (0.49–1.40)	11/20	11/20
6.4 (2.9–14.4)	5/5	5/5	5,6 (2.6–11.8)	5/5	5/5

The methods POD values and respective 95% confidence intervals (LCL, UCL) were calculated as the number of positive outcomes divided by the total number of trials, and, subsequently, the difference in the performance of the PNA-FISH method and the reference method, dPOD (C, R), and respective 95% confidence intervals (LCL, UCL) were calculated according Appendix J of the AOAC Official Methods of Analysis Manual for paired studies (AOAC, 2012; "Validation of Biomode S.A. Probe4Cronobacter TM for the Identification of Cronobacter spp. By AOAC Research Institute,"). As expected, equal POD values and dPOD (C, R) values of 0.00 (95% CI: 0.00; 0.00) were obtained for both food matrices. Thus, the POD analysis confirms that there is no statistically significant difference at the 5% level between the PNA-FISH method and the reference method for the detection of Campylobacter in fresh raw broiler meat and fresh raw ground pork (detailed results of the POD analyses are presented in Tables S4–S6).

3.6. Ruggedness test

The analysis of the robustness study shows that the variation of the selected parameters significantly affects the performance of the PNA-FISH method. The dPOD values analysis between the non-standard conditions and the baseline showed that lower hybridization time and temperature may affect the performance of method. Furthermore, the confidence interval of the $dPOD_{CB}$ values also showed significant differences in the performance of the PNA-FISH method between the combinations tested and baseline conditions, except for combinations 5 and 8 of the target strain. The accuracy values calculated were all low for the combinations tested (Table 6), including for combinations 5 and 8 of the target strain which previously have not shown dPOD significant differences from the baseline. This demonstrate that POD analysis alone can be limited, as results clearly show that all the combinations tested have a marked negative effect in the performance of the PNA-FISH method. The difference observed between POD analysis and accuracy analysis is a consequence of false positive and false negative results for each combination tested in comparison with the baseline.

The samples inoculated with *E. coli* CECT 515 had positive results (Table 6) in all conditions. In conditions with hybridization time and the temperature was lower than in the baseline, was observed 10 out of 10 positive samples, and the confidence levels for the dPOD values have shown significant differences. Using the combination When the hybridization time was lower and the temperature highest than in the baseline, the samples positives decreased to 6 (out of a total of 10) with significant differences too. These results seems to be associated with the addition of the blocker probe into the hybridization solution. The blocker probe has a perfect match with *E. coli* and *Salmonella*, thus preventing the binding of the mismatched probe. Thus, it seems that, with the increase of the hybridization temperature, the blocker probe loses the blocking effect that prevents nonspecific binding of the *Campylobacter* probe.

Similarly, to what was observed in the samples inoculated with *C. jejuni* NCTC 11168, the variations on time and hybridization temperature may result in false positive results. These deviations in the performance of the method indicate that the temperature and hybridization time are a key factor and should very tightly controlled, as small deviations can have a significant impact on method performance.

4. Conclusions

In this study, a PNA-FISH methodology, including enrichment culture, was optimized for detecting *Campylobacter* spp. in food samples. New PNA probe sequences were designed using updated 16S and 23S rRNA sequence databases. In order to be able to detect 1 CFU of *Campylobacter* spp. in 25 g of sample, different enrichment steps were

Table 6
PNA-FISH results and POD statistics analysis for the ruggedness study in fresh raw broiler meat artificially contaminated with *C. jejuni* NCTC 11168 and *E. coli* CECT 515.

***C. jejuni* NCTC 11168**

Combination	N[a]	X[b]	POD_{NT}[d]	95% CI[e]	$dPOD_{CB}$[f]	95% CI[e]	Accuracy[g]
1	10	10	1.00	0.72; 1.00	0.50	0.37; 0.63	50%
2	10	10	1.00	0.72; 1.00	0.50	0.37; 0.63	50%
3	10	9	0.90	0.60; 0.83	0.40	0.28; 0.52	60%
4	10	10	1.00	0.72; 1.00	0.50	0.37; 0.63	50%
5	10	6	0.60	0.31; 0.83	0.10	−0.08; 0.28	50%
6	10	10	1.00	0.72; 1.00	0.50	0.37; 0.63	50%
7	10	10	1.00	0.72; 1.00	0.50	0.37; 0.63	50%
8	10	6	0.60	0.31; 0.83	0.10	−0.11; 0.31	30%
9 (Baseline)	10	5	0.50	0.24; 0.76	/	/	100%

***E. coli* CECT 515**

Combination	N[a]	X[b]	POD_{NT}[d]	95% CI[e]	$dPOD_{CB}$[f]	95% CI[e]	Accuracy[g]
1	10	9	0.90	0.60; 0.83	0.90	0.82; 0.98	10%
2	10	10	1.00	0.72; 1.00	1.00	1.00; 1.00	0%
3	10	6	0.60	0.31; 0.83	0.60	0.48; 0.72	40%
4	10	6	0.60	0.31; 0.83	0.60	0.48; 0.72	40%
5	10	8	0.30	0.11; 0.60	0.80	0.70; 0.90	20%
6	10	10	1.00	0.72; 1.00	1.00	1.00; 1.00	0%
7	10	9	0.90	0.60; 0.83	0.90	0.82; 0.98	10%
8	10	6	0.60	0.31; 0.83	0.60	0.48; 0.72	40%
9 (Baseline)	10	0	0.00	0.00; 0.28	/	/	100%

c. PODT = Positive outcomes divided by the total number of trials with target C. jejuni NCTC 11168 for the correspondent condition.
[a] N = Number of tests.
[b] X = Number of positive tests.
[d] POD_{NT} = Positive outcomes divided by the total number of trials with non-target *E. coli* CECT 515 for the correspondent condition.
[e] 95% CI = Range of POD/dPOD values with a 95% confidence level.
[f] $dPOD_{CB}$ = Difference between the condition analysed (C) and the baseline (B) POD values. If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.
[g] Accuracy = Percentage of PNA-FISH results (either positive or negative) that corresponds to true results according to the baseline.

evaluated. The best enrichment step comprises: an incubation with Bolton broth for 4 h at 37 °C plus 44 h at 41.5 °C in microaerophilia (i.e. 5% O_2, 10% CO_2, and 85% N_2). After incubation, 1 mL of the enriched suspension should be centrifuged at 10 000 g for 5 min and the pellet resuspended in a 0.1% Tween-80 solution to decrease the auto-fluorescence conferred by the matrix compounds. The inclusivity and exclusivity study revealed a sensitivity of 92.0% and a specificity of 96.9% for the PNA-FISH method. Also, this method shows a performance similar to the ISO 10272-1:2017 reference method for two food matrices, fresh raw broiler meat and fresh raw pork. On the other hand, the ruggedness test showed that the temperature and hybridization time is a key factor and should very tightly be controlled, in order to guarantee the performance of the method.

The developed PNA-FISH method for detecting *Campylobacter* spp. in food samples is a simple and reliable method, providing results in less than 3 h after a 48 h enrichment step. In contrast, the ISO 10272-1:2017 method requires an additional 48 h to obtain confirmatory culture results. Alternatively, PCR can now be applied as molecular confirmation of the ISO culture procedure. Nevertheless, PNA-FISH is also comparable in time to other molecular methods such as PCR (2–3 h). The main limitation of PNA-FISH remains the need for a specialized fluorescence microcopy instrument.However, as it only detects viable cells, PNA-FISH represents a suitable alternative method for detecting *Campylobacter* spp. and/or as a confirmatory method to traditional standard methodology.

CRediT authorship contribution statement

Ricardo Oliveira: Investigation, Methodology, Writing – original draft. **Ana Barbosa:** Writing – original draft. **Mário Sousa:** Data curation, Validation. **Nuno Filipe Azevedo:** Supervision, Validation, Writing – review & editing. **Laura Cerqueira:** Data curation, Supervision, Writing – review & editing. **Carina Almeida:** Supervision, Validation, Writing – review & editing.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2024.115922.

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