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FISH DETECTION OF *CAMPYLOBACTER* AND *ARCOBACTER* ADHERED TO STAINLESS STEEL COUPONS

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ABSTRACT

This study focuses on detecting biofilm and planktonic bacterial cells of the genera *Arcobacter* and *Campylobacter*. This study is, to our knowledge, the first study deals with application of FISH procedure to detect biofilm formation on stainless steel coupons of *Arcobacter*-isolates from real-world environments. These bacteria can cause a lot of diseases. Especially, in the last decade, arcobacters have been increasingly isolated from feces of clinically healthy and ill animals, foods of animal origin and various types of water. Fluorescence *in situ* hybridization was used to detect biofilm and planktonic cells of selected microorganisms. This method was optimized and subsequently applied to biofilm samples prepared on stainless steel coupons. The study results indicate that fluorescence *in situ* hybridization is suitable for detecting biofilm and planktonic cells of the studied bacteria.

Keywords: FISH, biofilm, *Campylobacter*, *Arcobacter*, stainless steel coupons

INTRODUCTION

The genus *Arcobacter* arose through a relatively recent separation from the genus *Campylobacter*. Both of these genera are characterized by motile gram-negative rods and they are differentiated primarily based on growth conditions **(Douidah *et al.*, 2014; Figueras *et al.*, 2014)**.
These bacteria occur in water, the digestive tracts of domestic and wild animals and birds, foods of animal origin, and seafood. *Arcobacter* (*A.*) *butzleri*, *A. cryaerophilus*, *A. skirrowii*, *Campylobacter* (*C.*) *jejuni* and *C. coli* are most frequently connected with gastrointestinal disorders in humans and animals. As these bacteria are widespread among animals, the main routes through which humans become infected are through contaminated food, milk or water. These bacteria can cause bacteraemia, endocarditis, persistent watery diarrhoea in humans and septic abortions, mastitis and gastrointestinal disorders in animals **(Poppert *et al.*, 2008; Ahmed and Balamurugan, 2013; Ferreira *et al.*, 2013; Douidah *et al.*, 2014; *Figueras et al.*, 2014)**. These pathogens can form microbial communities known as biofilms. Biofilms are organized systems of cells growing on such various surfaces which have been exposed to a moist environment. Microbes form biofilms for many reasons, including easier exchange of generic material among the microorganisms and transfer of nutrients that are much more readily available in an aqueous state. If a biofilm is created in the food-processing industry, the cells can be released and thereby contaminate the product and subsequently the consumer **(Chmielewski and Frank, 2003; Trachoo, 2003; Lindsay and Von Holy, 2006; Sanz-Lázaro *et al.*, 2011; Ferreira *et al.*, 2013)**.
Microorganisms bound to a surface that is in contact with food constitute a potential hazard. Therefore, it is important to detect such microbes. Fluorescence *in situ* hybridization (FISH) is a combination of cytogenetic and molecular-genetic methods that is based on binding a fluorescent probe to the nucleic acid of the examined sample. Hybridization *in situ* is performed so that the probe marks the cell in the sample **(Moter and Göbel, 2000; Aoi, 2002; Zwirglmaier, 2005; Poppert *et al.*, 2008; Wagner and Haider, 2012; Pantanella *et al.*, 2013)**.
This technique helps to reveal the mechanisms of cell survival in the biofilm stage and is commonly used for detecting specific bacterial groups in mixed populations from various environments. The great advantages of the FISH method include that it enables direct determination of the number of bacteria using a fluorescence microscope, also allows determine the morphological characteristics of targeted micro-organisms, their cell size and cellular rRNA content. FISH is a very straightforward and powerful physical mapping technique which plays an increasingly important role in the study of genome structure and functional organization. It was developed to overcome the shortcomings of culture methods and methods based on polymerase chain reaction (PCR). Overall, FISH is a surprisingly simple and robust technique which has applications in many different research areas **(Fan *et al.*, 1990; Aoi, 2002; Zwirglmaier, 2005; Haaf, 2006; Fera *et al.*, 2010; Wagner and Haider, 2012)**. This study focuses on using a FISH method to detect planktonic cells and cells bound in a bacterial biofilm for representatives of the genera *Arcobacter* and *Campylobacter*. The objectives of the study were to develop a FISH method for selected bacteria and then apply the optimized method to prepared biofilm samples on stainless steel coupons (the most widely used material in the food industry).

MATERIAL AND METHODS

The determination of selected microorganisms using FISH was based on studies by **Bemfohr *et al.* (1993)** and **Amann *et al.* (1995)**, although individual steps needed to be further optimized for the specific conditions of detecting *Arcobacter* and *Campylobacter* strains. The steps that were optimized were the cell suspension density for preparing the specimen, the method of fixing the sample, the time necessary for hybridizing the probe, and the specificity of the fluorescent probe for the genera *Arcobacter* and *Campylobacter*.

Bacterial strains and culture conditions

A. butzleri CCUG 30484 (Culture Collection, University of Göteborg, Sweden), *A. skirrowii* LMG 6621 (Belgian Co-ordinated Collection of Microorganisms, Ghent University, Belgium), *A. cryaerophilus* CCM 3933, *C. jejuni* CCM 6214, and *C. coli* CCM 7227 (Czech Collection of Microorganisms, Masaryk University, Czech Republic) were used to prepare microbial suspensions. The following isolates from real-world environments (Collection of Microorganisms, University of Pardubice, Czech Republic) were included: *A. butzleri* UPa 2013/7, an isolate from chicken neck; *A. cryaerophilus* UPa 2012/1, an isolate from sewage water; and *A. skirrowii* UPa 2013/34, an isolate from cow's teat. *Cronobacter* (*Cr.*) *muytjensii* ATCC 51329 (American Type Culture Collection, USA) was used as a negative control.
To prepare the bacterial mixtures, the cultures of *Arcobacter* strains were cultured for 48 h at 30 °C under aerobic conditions on tryptone soya agar growth medium (Himedia, India) and the cultures of *Campylobacter* strains were cultured for 48 h at 42 °C in an anaerobic environment on Campylosel agar growth medium (Biomerieux, France).

Prepared biofilm samples

Stainless steel coupons for the food industry (type 304 finish 2b; Terapol, Czech Republic) were first cleaned using ultra-fine brush and liquid detergent (P&G-Rakona, Czech Rebublic) and rinsed in distilled water before being soaked in 70% ethanol. The coupons (4 x 1 cm) were allowed to air dry and then wrapped in foil and autoclaved before use.
The biofilm sample was prepared by culturing the bacterial suspension (1 ml; 10^8 cfu.ml^{-1}) on stainless steel coupons in 9 ml of Casein-peptone soymeal-peptone (CASO) broth (Merck, Germany). The final density of cells in the broth was therefore 10^7 cfu.ml^{-1}. In this manner, two sets of biofilm samples of *A. butzleri* CCUG 30484, *A. cryaerophilus* CCM 3933, *C. jejuni* CCM 6214 and *C. coli* CCM 7227 were prepared. Culturing was at 25 °C for 78 h under aerobic or (for *Campylobacter* strains) microaerobic conditions. The test tubes with coupons were placed in a vertical position.

Oligonucleotide probes

For the FISH method, an *Arcobacter*-specific probe (*Arc94*^{Cy3}: 5´-TGC-GCC-ACT-TAG-CTG-ACA-3´), a *Campylobacter*-specific probe (*Catherm*^{Cy3}: 5´-GCC-CTA-AGC-GTC-CTT-CCA-3´) and an eubacterial probe (*EUB338*^{FAM}: 5´-GCT-GCC-TCC-CGT-AGG-AGT-3´) were used after being synthetized and marked with fluorescent cyanine dye Cy^3 (indocarbocyanine; red) or FAM (6-carboxyfluorescein; green) from Generi-Biotech (Czech Republic). The *EUB338*^{FAM} universal probe, complementary to a region of 16S rRNA of the domain *Bacteria*, was used as a positive control to detect all bacteria present in each sample **(Poppert *et al.*, 2008; Fera *et al.*, 2010)**.

Determining the specificity of the oligonucleotide probes with DAPI dye

In order to determine the specificity of the probes, *in situ* hybridization was performed on all tested bacterial cells that were fixed to a slide with epoxy coating (Marienfeld Superior, Germany) using relevant fluorescent probes. FISH was performed with all bacterial strains and fluorescent probes and in various combinations. This means that for *Arcobacter* detection, both fluorescent probes, *Arc94*^{Cy3} and *Catherm*^{Cy3}, were used. The same was done also for *Campylobacter* strains and for the negative control.
The amount of 1 ml of bacterial suspension in saline solution (0.85% water solution of NaCl) with density of 10^8 cfu.ml^{-1} was centrifuged in a micro test tube (16,500 g, 5 min, 21 °C) and the supernatant was removed. The amount of 500 µl of 2% formaldehyde was added to the sediment. After incubation for 24 h at 4 °C, the micro test tube tubes were stirred and centrifuged again (16,500 g, 5 min, 21 °C). After removing the supernatant, 1 ml of phosphate buffer (PBS, consisting of 0.1 mol.l^{-1} NaCl, 0.002 mol.l^{-1} KH_2PO_4, 0.008 mol.l^{-1} $Na_2HPO_4{\cdot}12H_2O$, and 0.003 mol.l^{-1} KCl) was added to the sediment, followed by centrifuging (16,500 g, 5 min, 21 °C) and a repeated removal of the supernatant. This procedure was repeated once again, and, with the third washing, 500 µl of a mixture of PBS and 100% ethanol (1:1 ratio) was added to the sediment. After the mixture was added, 5 µl of the suspension was transferred to a slide with an epoxy coating, and after drying at 46 °C it was subjected to 3 min of dehydration with 96% ethanol. After drying at 46 °C, 9 µl of hybridization buffer (0.09 mol.l^{-1} NaCl, 0.02 mol.l^{-1} Tris/HCl, and 30% formamide) and 1 µl of fluorescent probe were pipetted into each well.
The slide thusly prepared was placed into a hybridization chamber (46 °C, 2 h). After hybridization, the slide was placed for 20 min into wash buffer (0.02 mol.l^{-1} Tris/HCl; 0.1 mol.l^{-1} NaCl, 0.005 mol.l^{-1} ethylenediaminetetraacetic acid, 0.01% sodium dodecyl sulphate) tempered at 48 °C. After two washings with sterile distilled water and drying in darkness at room temperature, 5 µl of DAPI dye in a 1 µg.ml^{-1} concentration (4',6-diamidino-2-phenylindole; Sigma-Aldrich, USA) was added to the wells with the samples. The slide thusly prepared was dried in darkness at room temperature, washed twice, and dried again in darkness at room temperature. To ensure proper adhesion of the cover slip, CC/Mount (Sigma-Aldrich, USA) was dripped onto the dry slide. The samples thusly prepared were examined without immersion using a Nikon ECLIPSE 80i (Nikon, Japan) epi-fluorescence microscope and NIS-Elements Viewer 4.0 analysis software, NIS-Elements AR 4.00.01 64-bit imaging software, fluorescence filters (DAPI; FITC – fluorescein isothiocyanate; TRITC – tetramethylrhodamine isothiocyanate; GFP/FITC/LP – green fluorescent protein/fluorescein isothiocyanate/longpass), and a digital camera (Nikon DS-Qi1Mc or DS-Fi1).

Determining the specificity of the oligonucleotide probes with eubacterial probe

In determining the specificity of the probes with eubacterial probe, *in situ* hybridization was performed on all tested bacterial cells that were fixed to a slide with epoxy coating using relevant fluorescent probes in combination with universal eubacterial probe *EUB338*^{FAM}. FISH was performed with all bacterial strains and fluorescent probes and in various combinations. This means that for *Arcobacter* detection, both fluorescent probes, *Arc94*^{Cy3} and *Catherm*^{Cy3}, were used. The same was done also for *Campylobacter* strains and for the negative control.
The samples were prepared as in the section *"Determining the specificity of the oligonucleotide probes with DAPI dye"*.
After the mixture was added, 5 µl of the suspension was transferred to a slide with an epoxy coating, and after drying at 46 °C it was subjected to 3 min of dehydration with 96% ethanol. After drying at 46 °C, 8 µl of hybridization buffer, 1 µl of fluorescent specific probe and 1 µl *EUB338*^{FAM} probe were pipetted into each well.
The slide thusly prepared was placed into a hybridization chamber (46 °C, 2 h). After hybridization, the slide was placed for 20 min into wash buffer tempered at 48 °C. The slide thusly prepared was washed twice, and dried in darkness at room temperature. To ensure proper adhesion of the cover slip, CC/Mount was dripped onto the dry slide. The samples thusly prepared were examined without immersion using a epi-fluorescence microscope as in the section *"Determining the specificity of the oligonucleotide probes with DAPI dye"*.

Optimization of cell suspension density

Suitable densities of the bacterial suspension for preparing the specimen (10^6–10^8 cfu.ml^{-1}) were tested and determined for all selected microorganisms.
The procedure then continued as in the sections *"Determining the specificity of the oligonucleotide probes with DAPI dye"* and "*Determining the specificity of the oligonucleotide probes with eubacterial probe"*.

Optimization of sample fixation

The next step in the optimization was the method of sample fixation – formaldehyde vs. heat. To prepare bacterial suspension with a density of 10^7 cfu.ml^{-1}, PBS and the bacterial strain *A. butzleri* CCUG 30484 were used.
For fixation by heat, 1 ml of the suspension was transferred to the micro test tube, then placed in dry heat at 110 °C for 15 min and subsequently cooled. For fixing with formaldehyde, 1 ml of bacterial suspension was transferred to the micro test tube and centrifuged (16,500 g, 3 min, 21 °C). After centrifuging, the supernatant was removed and 500 µl of 2% formaldehyde solution was added to the sediment. The sample thusly prepared was homogenized and placed into a refrigerator (4 °C, 24 h).
The samples were then processed as in the sections *"Determining the specificity of the oligonucleotide probes with DAPI dye"* and "*Determining the specificity of the oligonucleotide probes with eubacterial probe"*.

Optimization of the fluorescent probe's hybridization time

An appropriate time for hybridization of the fluorescent probe was tested for the strain *A. butzleri* CCUG 30484. The amount of 1 ml of bacterial suspension with density of 10^7 cfu.ml^{-1} was pipetted into a micro test tube. The tube was centrifuged (16,500 g, 5 min, 21 °C) and the supernatant removed. The amount of 500 µl of 2% formaldehyde was added to the sediment. This was followed by 24 h of incubation at 4 °C.
The samples were then prepared as in the sections *"Determining the specificity of the oligonucleotide probes with DAPI dye"* and "*Determining the specificity of the oligonucleotide probes with eubacterial probe"* with the exception of probe hybridization, which was carried out at 46 °C for 1, 2, 3 and 6 h.

Application of optimized methods on prepared biofilm samples

The test tubes with coupons were mixed, and then the coupons were removed and rinsed with sterile distilled water (2 ml for each side). The coupons were subsequently wiped with a sterile swab (each side 10 times). Swabs with the biofilm were shaken in 10 ml of PBS (2 min, vortex). The amount of 1 ml of the suspension in the PBS was pipetted into a micro test tube, which was then centrifuged (16,500 g, 5 min, 21 °C) and the supernatant removed. The amount of 500 µl of 2% formaldehyde was added to the sediment, followed by 24 h incubation at 4 °C.
For the second set of test tubes, after removing the biofilm the wiping swabs were shaken out into 10 ml of CASO broth. After culturing at the optimal temperature for each of the microorganisms (24 h for multiplication of the cells from the biofilm), the test tubes were stirred and 1 ml of each suspension was pipetted into micro test tubes that were subsequently centrifuged (16,500 g, 5 min, 21 °C). The supernatant was removed and 500 µl of 2% formaldehyde was added to the sediment. The samples thusly prepared were placed into a refrigerator at 4 °C for 24 h.
The samples were then processed identically as in the sections *"Determining the specificity of the oligonucleotide probes with DAPI dye"* and "*Determining the specificity of the oligonucleotide probes with eubacterial probe"* with the exception of hybridization, which was performed for 3 h. The samples thusly prepared were examined without immersion using an epi-fluorescence microscope.

RESULTS AND DISCUSSION

In the present study, a FISH method was introduced and optimized for detecting *Campylobacter* and *Arcobacter* cells (isolates from real-world environments) in prepared biofilms on stainless steel coupons.

Determining the specificity of the oligonucleotide probes with DAPI dye or with eubacterial probe

Specificity was determined for the $Arc94^{Cy3}$ probe for *Arcobacter* strains and the $Catherm^{Cy3}$ probe for *Campylobacter* strains. Both of the probes demonstrated specificity by the fact that the $Arc94^{Cy3}$ probe bound only to *Arcobacter* strains and the $Catherm^{Cy3}$ probe only to *Campylobacter* strains (data not shown). Neither of the probes bound to the strain *Cr. muytjensii* ATTC 51329 which was used as the negative control. The eubacterial probe $EUB338^{FAM}$ created a bond with all used strains of microorganisms, including the strain *Cr. muytjensii*.
A study by **Fera *et al.* (2010)** detected the genus *Arcobacter* in samples from river estuaries in southern Italy and also compared the PCR and FISH methods. They found both techniques to be suitable for detecting the genus *Arcobacter*. They confirmed the specificity of the primers used and that successful use of the hybridization probe is possible. Still earlier, **Moreno *et al.* (2003)** had worked on detecting the genera *Arcobacter* and *Campylobacter* in samples from river and sewage water using FISH.

Optimization of cell suspension density

The tested cell density of 10^8 cfu.ml^{-1} (Figure 1) was too high for application onto the slide, as the coating was too thick for microscopy and the cells formed into clumps. For this reason, the DAPI dye and fluorescent probes were washed out less successfully. This interfered with detection and increased background noise. Conversely, cell suspension density of 10^6 cfu.ml^{-1} (data not shown) proved too low for detection. Field of view contained only a few isolated cells, which may make it difficult to detect under a microscope. The tested cell density of 10^7 cfu.ml^{-1} was suitable for fluorescent *in situ* hybridization. The cells did not form clumps and the non-bound DAPI dye and fluorescent probes were washed out sufficiently (Figure 2).

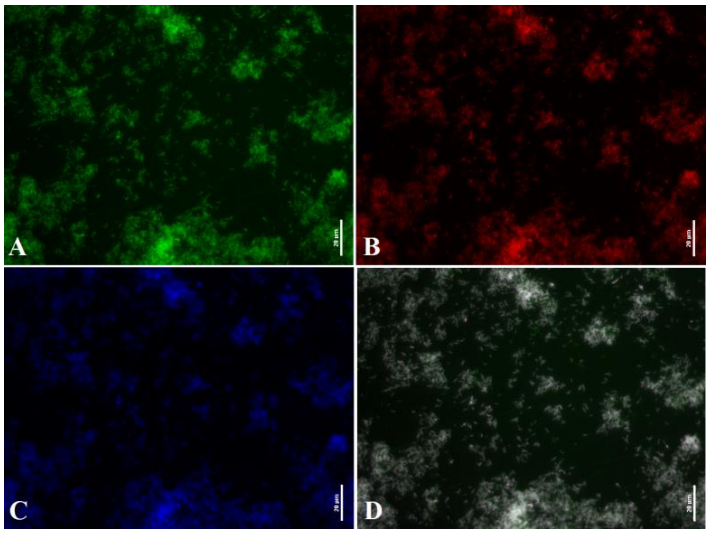

Figure 1 *Arcobacter butzleri* CCUG 30484 cells in the visual field epi-fluorescence microscope (cell density 10^8 cfu.ml^{-1}; scale 20 µm; A - cells with the bound $EUB338^{FAM}$ fluorescent probe; B - cells with the bound $Arc94^{Cy3}$ fluorescent probe; C – cells stained with DAPI dye; D - overlap of the visual fields A, B and C)

A similar method was employed in an earlier study by **Schauer *et al.* (2012)**, where the genus *Vibrio* was detected in water samples using the CARD-FISH method (catalyzed reporter deposition FISH). Optimal cell density was in that case defined as 10^7 and 10^6 cfu.ml^{-1}. Identically, in the present study optimal cell suspension density was 10^7 cfu.ml^{-1}.

Optimization of sample fixation

Samples for the FISH method were fixed using dry heat and 2% formaldehyde (data not shown). Heat fixation at 110 °C for 15 min should have ensured disruption of the cell membrane and that the fluorescent probes could bind to DNA. Using this method, the DAPI dye bound without complications, but the appropriate fluorescent probes did not, and therefore this method of cell fixation was deemed to be unsuitable. When fixation was performed with a 2% solution of formaldehyde, the DAPI dye again bound easily and the fluorescent probes also bound correctly.

Poppert *et al.* (2008) used FISH to detect thermotolerant strains of the genera *Campylobacter*, *Arcobacter* and *Helicobacter*. Cell fixation was not performed on the entire sample volume within a micro test tube, however, but rather on a microscope slide where the cell suspension was left to dry at laboratory temperature. The slides thusly prepared were placed into 2% formaldehyde for 20 min. As in the present study, a 2% solution of formaldehyde was used for fixation, but fixation was performed under different conditions. In contrast to the study by **Poppert *et al.* (2008)**, in the present study cell fixation was performed in micro test tubes with a sample volume of 500 µl. Fixation directly on a slide, where 5 µl of cell suspension suffices, can result in a noticeable shortening of fixation time.
Chen *et al.* (2008) published a study dealing with detecting *Heterosigma akashiwo* using FISH. Fixation was not performed with a solution of only paraformaldehyde or formaldehyde, however, but rather a mixture of 4% paraformaldehyde, 4% formaldehyde, 10% formalin and 2.5% glutaraldehyde. Subsequent fixation was then performed for only 5 min, which resulted in a noticeable shortening of the entire FISH process.

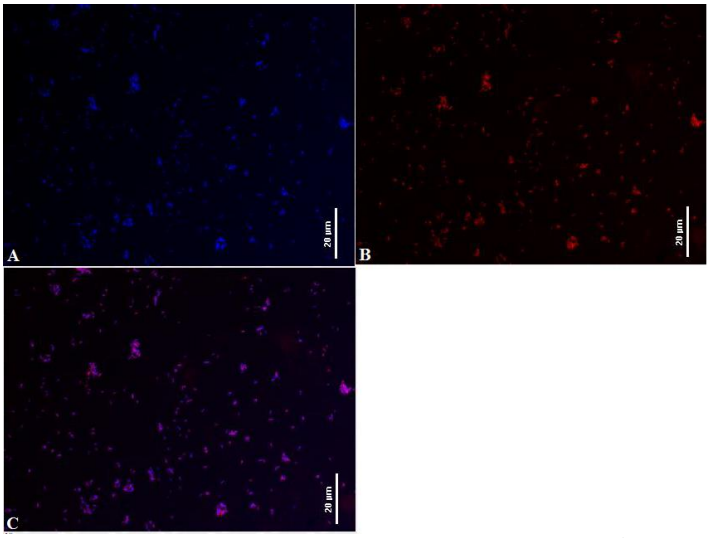

Figure 2 *Campylobacter jejuni* CCM 6214 cells in the visual field epi-fluorescence microscope (cell density 10^7 cfu.ml^{-1}; scale 20 µm; A - cells stained with DAPI dye; B - cells with the bound $Catherm^{Cy3}$ fluorescent probe; C – overlap of the visual fields A and B)

Optimization of the fluorescent probe´s hybridization time

Hybridization time in the steam of the hybridization solution is important and most frequently ranges between 1 and 6 h. Hybridization time at 46 °C was optimized based on testing at 1, 2, 3 and 6 h (data not shown).
Using microscopic detection, hybridization for 1 h was evaluated as insufficient for the binding of the fluorescent probe to the cell DNA for strains of both *Arcobacter* and *Campylobacter*. Hybridization for 2 h brought satisfactory results if a lower cell density was used. If the density of the microbial suspension was greater than 10^6 cfu.ml^{-1}, the signal of the fluorescent probe was diminished. With hybridization time at 3 h, detection distinctly improved even for samples with higher cell density. Prolonging the hybridization duration further (6 h) did not prove useful. The duration of 3 h at 46 °C was selected as the optimal hybridization time. Specific binding of the probes occurs within that time, and that is the priority for detection.
Despite stated possibilities as to hybridization time in the range of 8–24 h for optimal binding of fluorescent probes, hybridization time was not prolonged in the present study, as an overly long hybridization time can cause non-specific binding of the probe and less successful washing out of unbound dye.
In study by **Schauer *et al.* (2012)**, where the genus Vibrio was detected in water samples using the CARD-FISH method hybridization was then performed at 46 °C for 1.5–2 h. Identically, in the present study optimal cell suspension density was 10^7 cfu.ml^{-1}, and the hybridization necessary for binding of the fluorescent probe also was performed under the same conditions. As a result of the higher concentration of paraformaldehyde in the study by **Schauer *et al.* (2012)**, the time needed for cell fixation was decreased.

Application of optimized methods on prepared biofilm samples

In detecting biofilm in the samples prepared without prior enrichment, no microscopic response was observed (data not shown), as cell density with only this processing is too low. As shown in Figure 3 and Figure 4, 24 h of cell multiplication is necessary before detection proper.
Kofoed *et al.* (2012) used a FISH method to detect cells of *Pseudomonas stutzeri* in an artificially prepared biofilm sample. In order to enable biofilm detection, the cells were first multiplied overnight in an appropriate medium and then

detected using FISH. As in the present study, cell fixation was with 2% formaldehyde in a sample prepared in PBS. Micro test tubes with samples were incubated at 5 °C for 24 h.

Machado *et al.* (2013) used a PNA–FISH method (FISH using a peptide nucleic acid probe) to detect biofilms of *Lactobacillus* that had been cultured on plates for 24 h under anaerobic conditions at 30 °C or 37 °C. Both fixation methods were successfully used: fixation on a microscope slide and fixation in cell suspension (4% paraformaldehyde, 1 h, 4 °C). Fixation in suspension is generally used to prevent autofluorescence on the slide. Hybridization was performed at 60 °C for 90 min and washing was in a wash buffer tempered at 60 °C for 30 min. The study positively evaluated the fact that the entire detection process using the FISH method can be performed within 3 h.

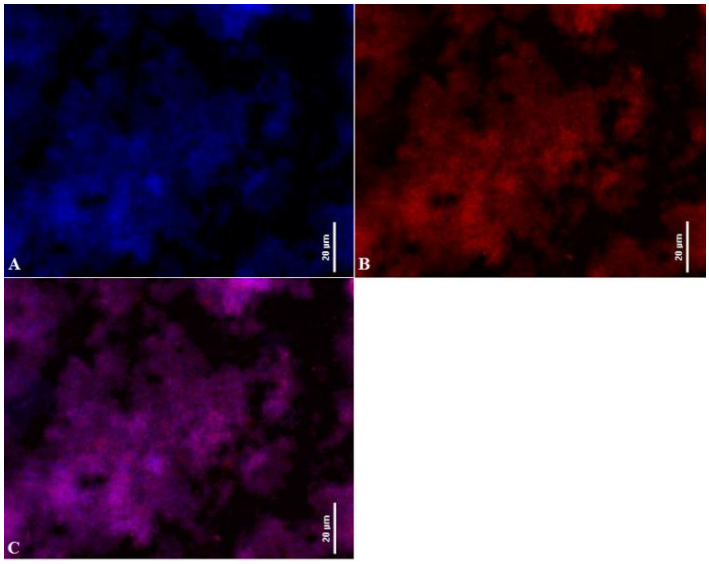

Figure 3 *Arcobacter butzleri* CCUG 30484 biofilm cells in the visual field epi-fluorescence microscope (scale 20 µm; A - cells stained with DAPI dye; B - cells with the bound *Arc94*^{Cy3} fluorescent probe; C – overlap of the visual fields A and B)

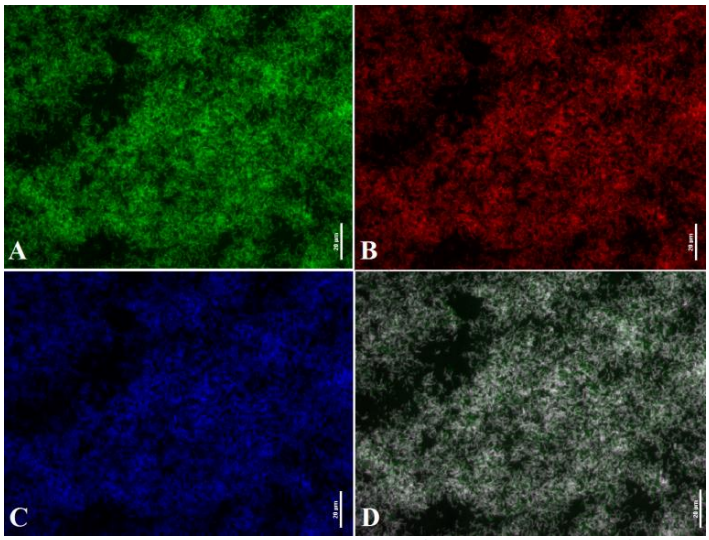

Figure 4 *Arcobacter butzleri* CCUG 30484 biofilm cells in the visual field epi-fluorescence microscope (scale 20 µm; A - cells with the bound *EUB338*^{FAM} fluorescent probe; B - cells with the bound *Arc94*^{Cy3} fluorescent probe; C – cells stained with DAPI dye; D - overlap of the visual fields A, B and C)

Comparison of FISH method with DAPI dye and FISH method with eubacterial probe

Fluorescent *in situ* hybridization was performed with DAPI dye or with universal fluorescent probe *EUB338*^{FAM} for domain *Bacteria*. Both methods have been applied to the samples of prepared biofilms. These methods are different, both in terms of time and in terms of performance.

The method with DAPI dye is time-consuming and more laborious. Moreover, the DAPI dye creates non-specific bond with the all genetic material and dye was worse removed from the sample during washing. However, the eubacterial probe creates specific binding to bacterial cells, the experimental time is reduced and after our experiments we can say that method using fluorescent eubacterial probe *EUB338*^{FAM} is more suitable in comparison with DAPI procedure.

CONCLUSION

Even though cells must be viable for detection using FISH, this method is considered more suitable than are methods using PCR. FISH is more stable against the influences of inhibitors and works independently of any enzymatic cell activity. This study has demonstrated that the FISH method can be successfully applied to biofilms and is therefore suitable for detecting biofilm. Both FISH techniques were suitable tools for identification of arcobacters and campylobacters confirming the excellent specificity of the primers used and the discriminatory power of the hybridization probe assay. The optimal density of cells for microscopic observation in this case appears to be density of 10^7 cfu.ml^{-1}. Biofilm observation is currently up to date, especially in the food industry. This method can be used as a convenient tool for the detection of biofilm on surfaces of different materials which are often used in the food industry.

This study deals with the single-species biofilm. The environment, however, there are also multi-species biofilms. A good idea in the future could therefore be multi-species biofilms detection, using multicolor fluorescence *in situ* hybridization.

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