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# Specific detection of bacteria from food using a combination of PEF and Flow-FISH

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## 1. Introduction, Knowledge, Objectives

With rising health consciousness the consumption of fruit and vegetables has increased in recent years (De Roever, 1998; Rediers et al., 2009). Significant changes in lifestyle have led to changes in consumption trend resulting in a higher demand for fresh-cut, minimally processed fruits and vegetables. An increased number of foodborne diseases are associated with these ready-to-eat products (Little & Gillespie, 2008; Doyle & Erickson, 2008).
The automated and specific detection of pathogens is an important task in food microbiology. The use of fluorescently labelled oligonucleotide probes in flow cytometry (Flow-FISH) allows the specific and automated identification of microorganisms in pure culture and also in environmental samples (Amann et al., 1990; Wallner et al., 1993; Vesey et al., 1994; Davey, 2002; Tang et al., 2005). The 16S rRNA is the common target for determinative hybridisation probes (Amann et al., 1990). It was shown that fluorescently labelled oligonucleotide probes specific for 16S rRNA can be used to monitor growth of bacteria in pure or mixed populations, sewage, lake water, and activated sludge(Thomas et al., 1997). However, limitation of Flow-FISH is the hindered uptake of oligonucleotide probes by the cells.
The use of Flow-FISH for specific detection of pathogenic bacteria on food surfaces requires the removal of bacteria from these surfaces into a suspension. Due to a high autofluorescent potential of food ingredients the destructive removal of bacteria from food surfaces should be avoided to prevent false positive detection of bacteria by flow cytometry due to autofluorescent food particles.
The aim of this study was to develop a non-destructive and reliable removal of bacteria attached to food surfaces, firstly and secondly to optimise a Flow-FISH protocol. The potential of the application of pulsed electric fields (PEF) as a pre-treatment was investigated in order to evaluate its effect of subsequent uptake of probes and the hybridisation rate.

## 2. Material and Methods

### *Attachment and removal of bacteria*

Golden Delicious apples were chosen as food matrix. *E. coli* (DSM 1116), was harvested in the exponential growth phase. A bacteria suspension containing $1.08 \times 10^{10}$ *E. coli* $ml^{-1}$ in 0.05 M PBS was prepared using the Multisizer™ 3 Coulter Counter® (Beckman Coulter,

Krefeld, Germany). An area of 6.2 $cm^2$ was marked on the apple surface and 100 µl of the bacteria suspension was homogeneously applied on this area. Each experiment was performed in triplicate. The bacteria were allowed to attach to the surface for 19 h at room temperature before removal. To remove the bacteria a beaker containing 6.2 ml 0.05 M PBS and 10 glass beads was pressed on the marked area. The bacteria were then mechanically removed by shaking for 5 min. The total cell count of the bacteria suspension obtained was again determined using the Coulter Counter and subsequently Flow-FISH was performed as described below.

***Fixation of bacteria for Flow-FISH analysis***

*E. coli* was harvested in the exponential growth phase and fixed with 3.7 % formaldehyde over night at 4 °C (after Kepner & Pratt, 1994), to avoid a degradation of RNA by endogenic RNAse (Moter & Göbel, 2000). Subsequently, the formaldehyde was removed by centrifugation (8000 x *g*, 20 min, 20 °C) and the samples were stored in 50 % ethanol at -20 °C until further use.

***PEF treatment of bacteria***

The ethanol was removed from the bacteria by centrifugation (8000 x *g*, 20 min, 20 °C) and the bacterial pellet was re-suspended in PBS buffer (electrical conductivity: 0.28 mS/cm). PEF treatment was conducted using an electric field strength of 40 kV/cm and a treatment time of 1300 µs. Hybridisation was performed directly after PEF treatment.

***Hybridisation***

Treated samples were centrifuged (8000 x *g*, 20 min, 20 °C) and the pelleted material was re-suspended in hybridisation buffer (containing 50 % formamid). The oligonucleotide probe EUB338 labelled with AlexaFluor488 (Invitrogen, Frankfurt, Germany) was added to the sample using a concentration of 5 ng/µl and incubated for 2 h at 46 °C. Subsequently, the samples were centrifuged (8000 x *g*, 20 min, 20 °C) to stop the reaction and the pelleted material was re-suspended 0.05 M PBS. This step was repeated to remove excessive probes. To avoid false hybridisation oligonucleotide NON-EUB338 labelled with AlexaFluor488 (Invitrogen, Frankfurt, Germany) was added to the samples in instead of EUB338 and hybridisation procedures were conducted as described before.

***Flow cytometry***

Cytomics FC500 (Beckman Coulter, Deutschland) was used for flow cytometric measurements. The fluorescence of the probes was excited with a laser at a wavelength of 488 nm and the emission was measured using a photomultiplier and a band pass filter of 525 ± 25 nm. Each experiment was performed in triplicate and 10000 cells were measured for each triplicate.

## 3. Results

***Removal of bacteria***

$4.3x10^8$ *E. coli* $ml^{-1}$ were detected after applied removal procedure. Microscopic observations showed no apple fragments in the sample (data not shown).

***PEF-Flow-FISH***

The application of PEF before hybridisation led to a higher fluorescence intensity indicating a higher hybridisation rate (Fig. 1) in contrast to cells without pre-treatment with PEF. Cells without probes were also measured to adjust flow cytometer settings and to allow a distinction between fluorescent and non-fluorescent cells. False hybridisation after pre-treatment with PEF was excluded using nonsense probe NON-EUB338 (data not shown).

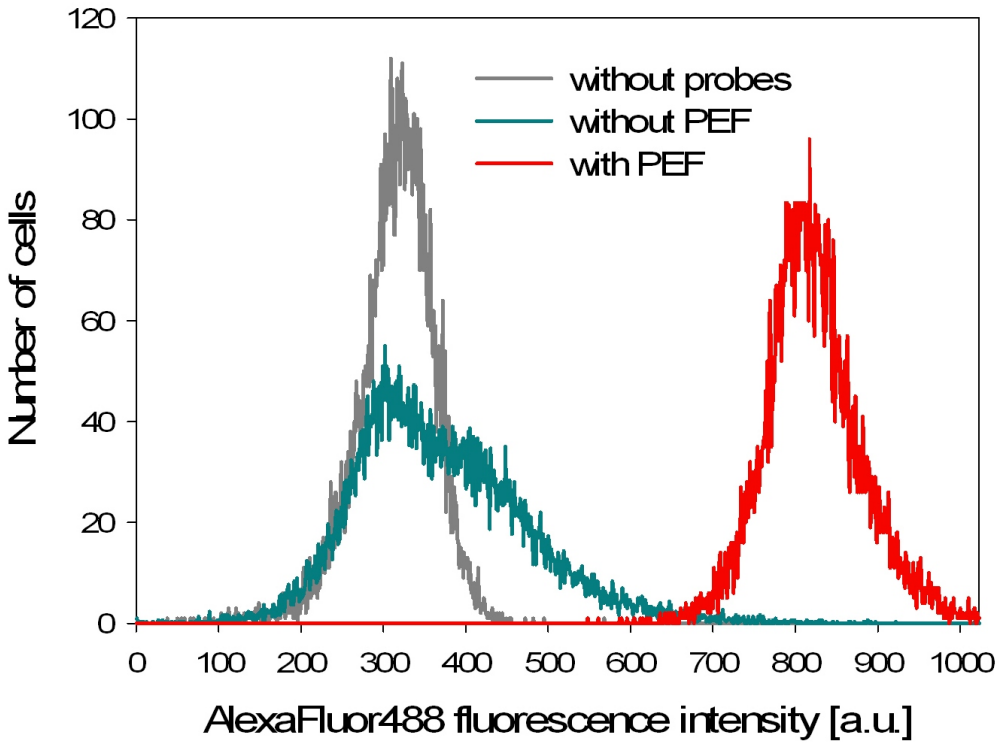


Figure 1: Hybridisation rate for *E. coli* using EUB338 labelled with AlexaFluor488 after PEF treatment in comparison to the hybridisation rate without PEF-treatment and *E. coli* cells without probes. Lines represent the average of three different measurements.

## 4. Discussion

Coulter Counter measurements showed that the bacteria could be non-destructively removed from the apple surface using the beaker and glass beads. A false positive detection due to autofluorescent apple particles within the suspension can be neglected.

The uptake of oligonucleotide probes can be realised using enzymatic or chemical permeabilisation steps with the risk of cell lysis (Zwirglmaier, 2005). However, chemical substances are unwanted in the food production chain due to potential hazardous contamination of products. Dehydration steps used to increase uptake of probes are time-consuming and therefore unsuitable for automatic detection of bacteria.

Electroporation uses electrical pulses to create pores in cell membranes which is known to improve the uptake of molecules such as nucleic acids and used in cell biology (Weaver, 2000). Reversible or irreversible cell permeabilisation can be induced by PEF depending on the applied treatment parameters (Knorr et al., 2011). The electrical field strength of 40 kV/cm was chosen to increase the uptake of probes because the applied electrical field strength led to an irreversible cell permeabilisation (Tsong, 1996).

## 5. Conclusions

With the developed method bacteria could be non-destructively and reliable removed from food surfaces. Automation of bacterial removal from food surfaces seems to be feasible with this preparation method.
PEF treatment highly improved the hybridisation rate of pure cultures allowing a reliable detection of bacteria by flow cytometry. These experiments indicate that the commonly applied dehydration step of bacteria before hybridisation that include several centrifugation steps can be replaced by PEF and this may facilitate the automation. The applicability of PEF-assisted Flow-FISH to other bacteria species as well as to bacteria from food samples will be tested in future experiments.

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