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ORIGINAL ARTICLE

Effect of pulsed light on structural and physiological properties of *Listeria innocua* and *Escherichia coli*

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Keywords
decontamination process, DNA damage, flow cytometry, pulsed light, RAPD-PCR, reactive oxygen species, viability staining.

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Abstract

Aims: The application of broad-spectrum intense light pulses is an innovative nonthermal technology for the decontamination of packaging materials, liquids or foodstuffs. The objective of this study was the fundamental investigation of the cellular impact of a pulsed light treatment on *Listeria innocua* and *Escherichia coli*.

Methods and Results: Flow cytometry in combination with different fluorescent stains, conventional plate count technique and a viability assay were applied to investigate the effects of a pulsed light treatment on the physiological properties of *L. innocua* and *E. coli*. The results showed that loss of cultivability occurred at considerably lower fluences than the shutdown of cellular functions such as the depolarization of cell membranes, the loss of metabolic, esterase and pump activities or the occurrence of membrane damage. Therefore, a considerable proportion of cells appeared to have entered the viable but nonculturable (VBNC) state after the pulsed light treatment. A high percentage of *L. innocua* was able to maintain certain cellular vitality functions after storage overnight, whereas a further decrease in vitality was observed in case of *E. coli*. The loss of culturability was on the other hand directly accompanied by the formation of reactive oxygen species (ROS) and DNA damages, which were assessed by the ROS-sensitive probe DCFH-DA and RAPD-PCR, respectively.

Conclusions: A significant discrepancy between conventional plate counts and different viability staining parameters was observed, which shows that a pulsed light treatment does not cause an immediate shutdown of vitality functions even when the number of colony-forming units already decreased for more than 6 $\log_{10}$ sample^{-1}. Oxidative stress with concomitant damage to the DNA molecule showed to be directly responsible for the loss of cultivability due to pulsed light rather than a direct rupture of cell membranes or inactivation of intracellular enzymes.

Significance and Impact of the Study: The presented results suggest an UV light-induced photochemical rather than a photothermal or photophysical inactivation of bacterial cells by pulsed light under the applied experimental conditions. Flow cytometry in combination with different viability stains proved to be a suitable technique to gain deeper insight into the cellular response of bacteria to inactivation processes like a pulsed light treatment.

Introduction

Recent developments among consumers regarding the demand for fresh, minimally processed foods with a preferably long shelf life have prompted the research for new and innovative technologies to ensure an appropriate preservation and safety of foodstuffs. The trend towards ready-to-eat products such as fresh-cut fruits or salads is accompanied by a rising public health threat due to foodborne diseases. An increasing number of listeriosis incidents in Germany during the last years may be attributable to this development (RKI 2010). Severe

outbreaks of foodborne diseases are frequently associated with contaminated raw vegetables or fruits, examples are the EHEC outbreak 2011 in Germany which was most likely attributable to sprouts (RKI 2011) or a multistate listeriosis outbreak in 2011 in the USA due to contaminated cantaloupe (CDC 2012).

Fresh-cut produce usually cannot be decontaminated by conventional heating treatments, and washing or sanitizing methods usually do not provide a sufficient reduction in microbial load (Sapers 2001). Therefore, nonthermal decontamination processes are needed to guarantee an adequate safety of fresh produce. Among various innovative strategies to inactivate micro-organisms such as the application of high pressure, gas plasma or pulsed electric fields, the pulsed light technology is an emerging technique for the decontamination of surfaces, for example packaging material or food products (Oms-oliu *et al.* 2010). A pulsed light treatment is a rather nonthermal process that involves short-duration light pulses of high power with a broad emission spectrum between 200 and 1100 nm (Elmnasser *et al.* 2007). Besides its advantages to be very fast and cost-effective, there are no residual compounds that have to be removed from foodstuff (Gómez-López *et al.* 2007). This technique has already been proven to be an efficient way for inactivating various pathogens and spoilage micro-organisms in or on different matrices (Rowan *et al.* 1999; Anderson *et al.* 2000; Gómez-López *et al.* 2005; Woodling and Moraru 2007; Farrell *et al.* 2009; Levy *et al.* 2012). The loss of culturability is thereby typically taken as single criteria for cell death, so usually no deeper insight into the physiology of pulsed light-treated microbial cells is obtained. However, flow cytometric investigations combined with certain fluorescent probes represent a suitable approach to gain useful information about different structural or functional properties of micro-organisms (Díaz *et al.* 2010; Müller and Nebe-von-Caron 2010; Sträuber and Müller 2010; Tracy *et al.* 2010). This technique can be used to study the cellular response of micro-organisms to stress situations like inactivation processes such as a pulsed light treatment (Kennedy *et al.* 2011; Nocker *et al.* 2011). In this way, flow cytometry may contribute to elucidate the mode of action of chemical or physical decontamination processes.

Only few studies have been dealing with the underlying inactivation mechanisms of pulsed light so far (Takeshita *et al.* 2003; Farrell *et al.* 2011; Cheigh *et al.* 2012). It is assumed that the UV component is the most important wavelength region for the bactericidal effect of pulsed light, as it is known that UV radiation affects the genome integrity. On the other hand, several studies also report on the disruption of microbial cells by pulsed light, so that it is believed to be a multi-target inactivation process with photochemical but also photothermal or photophysical effects being involved (Wekhof 2000; Takeshita *et al.* 2003; Krishnamurthy *et al.* 2008, Farrell *et al.* 2011; Cheigh *et al.* 2012). In this study, the cellular response of *Listeria innocua* and *Escherichia coli* on a pulsed light treatment was investigated. Therefore, the impact of pulsed light on the cultivability, metabolic activity, esterase activity, membrane potential, efflux pump activity and membrane integrity was studied as well as the formation of reactive oxygen species and the occurrence of DNA damages.

Material and methods

Bacterial strains, growth conditions and preparation of inoculums

The Gram-positive bacterium *L. innocua* DSM 20649, which is often used as a nonpathogenic surrogate for *Listeria monocytogenes*, was used as a model organism in this study, as well as the Gram-negative bacterium *E. coli* DSM 498. Both test strains were obtained from the German National Resource Center (DSMZ, Braunschweig, Germany). The bacteria were initially grown in 100 ml tryptic soy broth (Oxoid, Hampshire, UK) at 37°C for 16–18 h in a shaking bath. Tryptic soy agar (Oxoid) was subsequently inoculated with the bacterial suspension by use of an inoculating loop, incubated for 24 h at 37°C and stored at 5°C. Working cultures were made by inoculating 100 ml of tryptic soy broth with cell material from the agar surface and following incubation for 16–18 h at 37°C in a shaking bath until early stationary phase. 20 ml of the cell culture was subsequently centrifuged at 9000 ***g*** for 10 min and washed twice with sterile deionized water. The cell count was determined in a counting chamber by microscopic methods and finally adjusted to approximately 2×10^8 cells ml^{-1}.

The pulsed light treatments were conducted on the surface of the gellan gum Gelrite (Roth, Karlsruhe, Germany). It is an anionic heteropolysaccharide that forms clear and heat-stable gels in the presence of soluble salts. Therefore, 1% Gelrite with 0·1% $CaCl_2 \cdot 2\ H_20$ (VWR, Darmstadt, Germany) was boiled in sterile deionized water until it completely dissolved, and 15 ml was then immediately poured into Petri dishes. After solidification, discs with a diameter of 1·5 cm and a thickness of 2–3 mm were cut out and placed on glass slides. Three discs were used for each sample. The gel surface of each disc was inoculated with 50 μl of the prepared cell suspension and dried for 1·5–2 h in a safety cabinet at room temperature before treating the samples in the pulsed light chamber. The resulting cell density on the gel surface was about 3×10^6–1×10^7 cells cm^{-2}.

Pulsed light set-up and treatment conditions

The prepared samples were treated in a pulsed light chamber (Claranor, Avignon, France), which was equipped with a three xenon tubes reflector. The lamp is connected to a capacitor and emits broad-spectrum intense light flashes between 200 and 1100 nm. The applied voltages ranged from 1 to 3 kV, which corresponded to fluences between 0·10 and 1·08 J cm^{-2} at a distance of 10 cm from the reflector. The total energy input was determined with a SOLO2 Power and Energy Meter (Gentec, Quebec City, Canada). The UV contents of pulsed light at different voltages were determined by measuring the fluence on top of the detector head without and with a FGL400S longpass filter (Thorlabs, Newton, NJ) transmitting wavelengths over 400 nm only. The differences of the determined values gave approximately the respective UV content, the results are given in Table 1 expressed as percentages. Each sample was individually exposed to one single pulse, except when energy doses of more than 1·08 J cm^{-2} were applied. In this case, up to five pulses with a fluence of 1·08 J cm^{-2} were applied at a frequency of 1 Hz. Immediately after the treatment, the gel discs were transferred to polystyrene tubes (Greiner Bio-one) containing 5 ml of phosphate-buffered saline (PBS, pH 7·2; Gibco, Carlsbad, CA) that was previously filtered through a 0·22-μm pore size filter. Control samples were handled the same way, but without pulsed light treatments. The samples were agitated on a shaking device at 400 rev min^{-1} for 15 min to detach the bacteria from the gel surface. The total cell count of the sample suspension was then approximately 3×10^6–1×10^7 cells ml^{-1}.

Determination of colony-forming units (CFU)

The culturability of the PL-treated bacteria was determined by measuring the number of colony-forming units of each sample. 50 μl of appropriate dilutions in Ringer solution (Oxoid) or undiluted sample suspension was spread onto tryptic soy agar with an Eddy-Jet spiral plater (IUL instruments, Königswinter, Germany). Additionally, 1 ml of undiluted sample suspension was also investigated by the pour-plating method using tryptic soy agar. The plates were subsequently incubated for 24 h at 37°C, and the number of colony-forming units per sample determined with the Countermat Flash and Grow (IUL instruments, Königswinter, Germany). All samples were analysed immediately after the pulsed light treatment and again after a storage period of 24 h at 5°C. All samples were investigated in triplicates.

Table 1 Fluences in dependence from the applied voltage at a distance of 10 cm from the reflector with corresponding UV percentages

Voltage (kV)	Total fluence (J cm^{-2})	UV content (%)
1·0	0·10	5·8
1·5	0·27	9·8
2·0	0·50	12·3
2·5	0·78	14·3
3·0	1·08	17·5

Determination of metabolic activity

The metabolic activity of *L. innocua* and *E. coli* after the pulsed light treatment was determined with the Microbial Viability Assay kit (Dojindo Molecular Technologies, Kumamoto, Japan). This kit contains the tetrazolium salt WST-8 and the electron mediator 2-methyl-1·4-naphthoquinone. WST-8 is cleaved to a water-soluble formazan dye by active dehydrogenases, indicating metabolic activity of microbial cells. This reaction leads to the formation of a yellow colour at which the intensity is proportional to the number of viable cells (Tsukatani *et al.* 2008). 100 μl of the sample suspensions were transferred to a 96-well microplate and mixed with 100 μl twofold-concentrated Mueller–Hinton broth. 10 μl of a 1 : 10 (for *E. coli*) or 1 : 80 (for *L. innocua*) mixture of the electron mediator and WST-8 was added immediately. Each sample was measured in triplicates, and blanks (PBS) as well as untreated controls were included in every assay. The absorption was measured at 450 nm and recorded every minute in a microplate reader (Spectramax 190; Molecular Devices, Sunnyvale, CA) for 1 h at 30°C. Within this time frame, a linear increase in absorption could be observed. The relative metabolic activity after the pulsed light treatment was determined by calculating the ratio of absorbance between the treated samples and the untreated controls after 60 min. All samples were analysed immediately after the pulsed light treatment and again after a storage period of 24 h at 5°C.

Investigation of membrane integrity, membrane potential, esterase activity and pump activity by flow cytometry

The impact of the pulsed light treatment on the membrane integrity of cells was investigated on the basis of their staining characteristics using the fluorescent dye propidium iodide (PI, Dojindo Molecular Technologies, Kumamoto, Japan). Propidium iodide is a widely used indicator for membrane damages as it is a red fluorescent double-charged cationic molecule that intercalates in double-stranded DNA of compromised cells, but is usually excluded from cells with intact membranes (Sträuber and Müller 2010; Tracy *et al.* 2010). Permeabilized cells

are normally classified as dead because they are unable to maintain their intracellular environment (Hammes *et al.* 2011). An aliquot of 500 μl of each sample was incubated with 30 μmol l^{-1} PI for 10 min at room temperature in the dark before the flow cytometric analysis.

Depolarization of cell membranes was assessed with bis (1,3-dibutylbarbituric acid) trimethine oxonol, sodium salt ($DIBAC_4$, Dojindo Molecular Technologies, Kumamoto, Japan). This anionic lipophilic fluorescent dye accumulates in depolarized cells, but is excluded from cells that are able to maintain a transmembrane potential gradient. By investigating the membrane potential, it is possible to analyse the energetic state of a cell as it plays a key role in cell physiological processes (Sträuber and Müller 2010; Tracy *et al.* 2010). Cells without membrane potential cannot retain an intracellular environment that enables the functioning of metabolic processes, so membrane potential is in theory a good discriminator between living and dead cells (Hammes *et al.* 2011). 500 μl of each sample was incubated with 2 μmol l^{-1} (*L. innocua*) or 5 μmol l^{-1} (*E. coli*) $DIBAC_4$ and 5 g l^{-1} glucose for 15 min at room temperature in the dark before the flow cytometric analysis.

Bacteria contain a number of mechanisms that protect them against toxic compounds, for example antibiotics. One of the active and energy-dependant mechanisms is the expression of efflux pumps (Müller and Nebe-von-Caron 2010). The efflux pump activity of pulsed light-treated bacteria was investigated with 3·8-diamino-5-ethyl-6-phenylphenanthridinium bromide (EB; Sigma-Aldrich, St. Louis, MO). This fluorescent dye is actively extruded by several bacteria via efflux pumps, so this property can be considered as another energy-dependent vitality parameter (Hammes *et al.* 2011). Preliminary tests revealed that *L. innocua* does not show any efflux pump activity for this fluorescent dye, so the efflux capacity was only investigated for *E. coli*. 500 μl of sample suspension was incubated with 30 μmol l^{-1} EB and 5 g l^{-1} glucose for 15 min at 37°C in the dark before the flow cytometric analysis.

The esterase activity of pulsed light-treated bacteria was assessed with 5(6)-carboxyfluorescein diacetate (CFDA, Dojindo Molecular Technologies, Kumamoto, Japan). CFDA is an uncharged nonfluorescent lipophilic substrate that is hydrolysed to fluorescent carboxyfluorescein (CF) by unspecific esterases in the cytoplasm. The actual cleavage is energy independent; hence, the detection of enzyme activity is not necessarily equivalent to cell viability (Hammes *et al.* 2011). 500 μl of the sample suspension was incubated with 100 μmol l^{-1} CFDA for 15 min at 37°C in the dark. In case of *E. coli*, 1 mmol l^{-1} EDTA was added during the incubation period to facilitate the uptake of CFDA into the cells. The bacterial suspensions were subsequently centrifuged at 10 000 ***g*** for 5 min, and the cell pellets were resuspended in PBS before the flow cytometric analysis.

The flow cytometric analyses were performed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA), which is equipped with a 488 nm Argon laser. Bacteria were gated on the basis of the forward (FSC) and side scatter (SSC) signals. A total of 10 000 cells of each sample were analysed at a flow rate of 12 μl min^{-1}. The fluorescent signals of the cells were detected in the red channel FL3 (>670 nm) for PI or EB and in the green channel FL1 (515–545 nm) for CF or DIBAC4. Heat-treated cells (85°C, 10 min) served as negative controls for CFDA and positive controls for $DIBAC_4$, EB and PI staining. The data were recorded and analysed with CellQuest Pro software (Becton Dickinson). All samples were analysed within 2 h after the pulsed light treatment and again after a storage period of 24 h at 5°C.

Detection of reactive oxygen species (ROS)

To investigate an enhanced formation of reactive oxygen species in *L. innocua* due to a pulsed light treatment, the oxidative stress-sensitive probe 2′·7′-dichlorofluorescin diacetate (DCFH-DA) was used (Tracy *et al.* 2010; Farrell *et al.* 2011). DCFH-DA was obtained from Sigma-Aldrich and dissolved in dimethylsulfoxide (DMSO, VWR). The acetyl groups of DCFH-DA were removed by cellular esterase to form 2′·7′-dichlorodihydrofluorescein (DCFH). DCFH is nonfluorescent, but is highly fluorescent when oxidized to 2′·7′-dichlorofluorescein (DCF) by ROS (Farrell *et al.* 2011; Wang *et al.* 2012). Immediately after the PL treatment, the samples were incubated with 25 μmol l^{-1} DCFH-DA for 1 h at 37°C in the dark. Subsequently, the mean values of the fluorescent signals of 10 000 bacterial cells were recorded with a FACSCalibur flow cytometer at an excitation wavelength of 488 nm and a green emission filter FL1 (515–545 nm). The relative increase in fluorescence was determined by calculating the mean fluorescence ratio of treated samples to untreated references.

Detection of DNA damages by RAPD-PCR

To evaluate DNA damages with *L. innocua* caused by pulsed light, 2 ml of each sample suspension was initially centrifuged at 9000 ***g*** for 10 min. The cell pellet was resuspended in 180 μl lysis buffer (2 mmol l^{-1} EDTA, 20 mg ml^{-1} lysozyme, 20 mmol l^{-1} Tris pH 8, 1% Tween 80) and incubated for 30 min at room temperature. The genomic DNA was then extracted and purified with a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RAPD-PCR was performed using the illustra Ready-To-Go RAPD Analysis Kit (GE Healthcare, Buckinghamshire, UK)

according to the manufacturer's instructions. Three different primers (2, 5 and 6) were used to randomly amplify genomic DNA sequences of *L. innocua* (Table 2). Each RAPD analysis bead was mixed with 15 μl water, 5 μl primer (25 pmol) and 5 μl of extracted DNA. PCR was performed in a Rotor-Gene real-time PCR cycler (Qiagen) using the following profile: 1 cycle: 95°C for 5 min; 45 cycles: 95°C for 1 min; 36°C for 1 min; 72°C for 2 min. Separation of DNA fragments was carried out with a QIAxcel capillary gel electrophoresis equipped with a DNA high-resolution gel cartridge (Qiagen). To obtain a specific banding pattern, the method OM 500 was used in combination with a 15–5000 bp alignment marker and an intensity calibration marker. A DNA size marker ranging from 100 to 3000 bp was used to determine the sizes of DNA fragments. The generated DNA fingerprints of samples with different fluencies were compared to the band pattern of untreated cells for the three primers that were used. The total amount of DNA that was amplified by RAPD-PCR was determined with the software BioCalculator (Qiagen) on the basis of the band intensities. The ratios of the samples and the corresponding untreated controls were calculated, and the average relative amount of amplified DNA was determined on the basis of the data from the three primers.

Statistical analyses

Each test was performed on the basis of three replicate samples for each treatment condition from the same cell culture. Mean values and standard deviations were calculated. All tests were performed at least twice in the same way at different days with fresh cell cultures. Presented data are results from one representative trial. Statistically significant differences between treated samples and untreated controls were identified by one-way ANOVA using SigmaPlot 12·3 (Systat Software Inc., San Jose, CA) with the Holm-Sidak method at a significance level of $P < 0{\cdot}05$.

Results

Impact of pulsed light on colony count and metabolic activity

The impact of a pulsed light treatment on the cells' ability to form colonies on tryptic soy agar and their metabolic activity was assessed by conventional plate count technique and a viability assay, respectively. The following conclusions and statistical statements refer to both replicated cultures. No differences concerning their susceptibility to pulsed light could be observed between the two test bacteria *L. innocua* and *E. coli* by conventional plate counts. At fluences of more than 0·10 J cm^{-2}, a log-linear decrease in the viable cell count could be observed for both bacteria, and an inactivation of more than 6 $\log_{10}$ CFU sample^{-1} was achieved by one single light pulse at a voltage of 3 kV, which corresponded to a fluence of 1·08 J cm^{-2}. After the samples were stored for 24 h at 5°C and analysed again, almost the same results were obtained, so no recovery or progressive cell death could be observed in this case (Fig. 1). The reduction of WST-8 to a yellow formazan dye is indicative for microbial metabolism and therefore serves as a parameter for cellular viability. Despite a strong dose-dependent drop of the colony count due to the pulsed light treatment, the metabolic activity of *L. innocua* and *E. coli* decreased considerably slower (Fig. 2). At 0·10 J cm^{-2}, the metabolic activity of both bacteria was only marginally reduced, which is in accordance with the determined plate counts, where no statistically significant inactivation effect was evident at this fluence. With rising energy doses, the colony count decreased rapidly, however, statistically significant metabolic activity was still measurable up to an applied fluence of 1·08 J cm^{-2} with both bacteria. Relative metabolic activities of about 30–40% were determined after applying a fluence of 0·78 J cm^{-2}. This corresponds to an energy dose which caused a drop of the colony count for more than 99·99%, so there was no direct correlation between these two parameters in this case. When untreated references were twofold diluted, the relative metabolic activity was reduced to about 45% (Fig. 2), which shows that the colour development of the applied assay correlates very well with the number of respiring cells within the applied time frame of 60 min. These findings indicate that a considerable number of bacterial cells may still be metabolically active immediately after a pulsed light treatment although they have lost their capability to reproduce on a nutrient agar surface. In case of *L. innocua,* the metabolic activity also remained on a similar level after 24 h, whereas the relative metabolic activity of *E. coli* considerably decreased after the same storage time.

Table 2 Primers that were used for RAPD-PCR

Primer	Sequence
2	5′-d[GTTTCGCTCC]-3′
5	5′-d[AACGCGCAAC]-3′
6	5′-d[CCCGTCAGCA]-3′

Effect of pulsed light on cell physiology investigated by flow cytometry

To investigate the impact of a pulsed light treatment on functional and structural features of *L. innocua* and *E. coli* on a single cell level, flow cytometric analyses

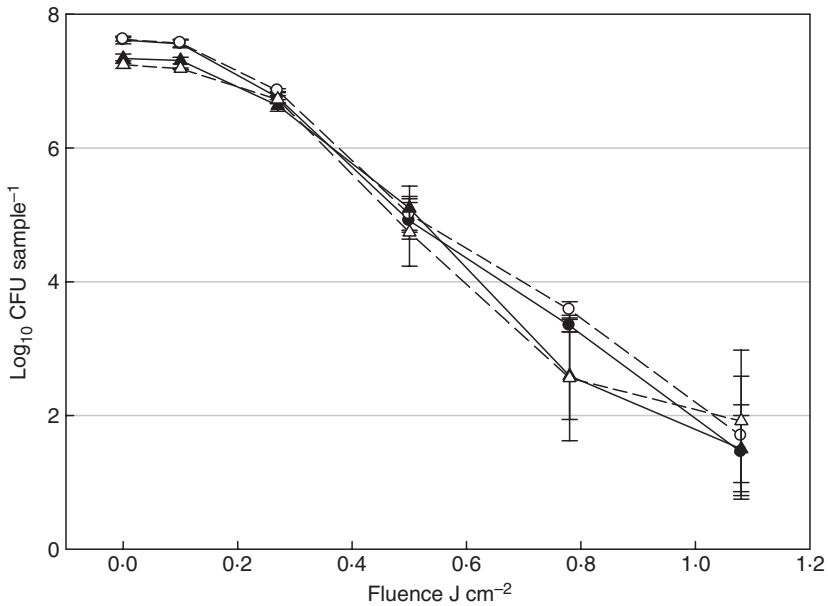

Figure 1 Inactivation kinetics of *Listeria innocua* and *Escherichia coli* determined by plate count method immediately after the treatment and after sample storage for 24 h at 5°C. The data represent mean values and standard deviations of triplicates. Samples: (●) *L. innocua* 0 h, (○) *L. innocua* 24 h, (▲) *E. coli* 0 h, (△) *E. coli* 24 h.

combined with several fluorescent dyes were performed immediately after the treatment and again after a storage period of 24 h at 5°C. The outcome of these investigations showed that there is no direct correlation between the results obtained by conventional plate count technique and those obtained by culture-independent viability staining methods. On the other hand, the obtained results of energy-dependant parameters such as membrane potential and efflux activity were in good agreement with the determined levels of metabolic activity for both bacteria. The decrease in metabolic activity was accordingly accompanied by the cessation of efflux pump activity and an increasing proportion of depolarized cells. The results for *L. innocua* and *E. coli* are summarized in Figs 3–5 and are representative for each replicated culture.

The uptake of PI, which is indicative for compromised cell membranes, was statistically significantly enhanced only at high fluencies of more than 3·24 J cm^{-2} with both test bacteria immediately after the treatment (Figs 3 and 4). After storage of the samples, about 10% of the untreated *L. innocua* population lost their membrane integrity and also a slightly higher percentage of *E. coli* cells were permeable for PI, but apart from that, the overall trend for this parameter did not change considerably. Membrane

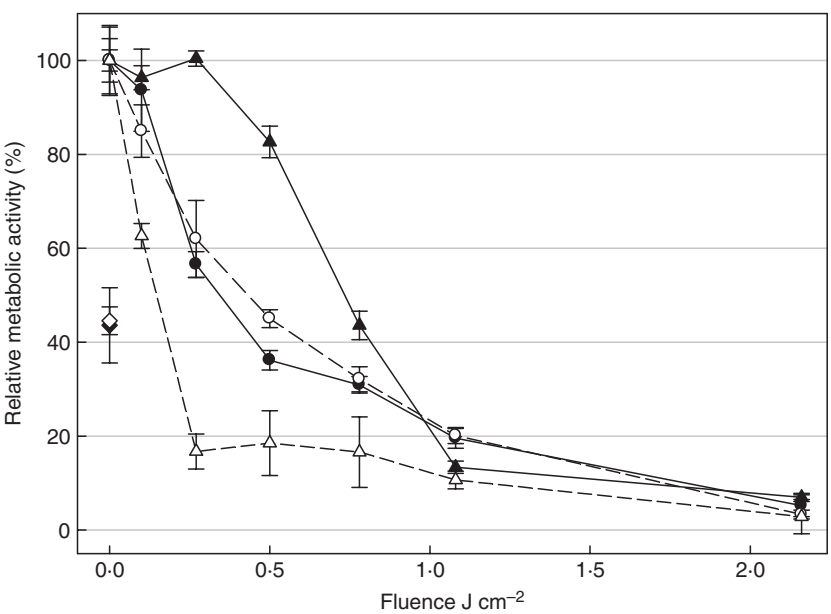

Figure 2 Relative metabolic activity of *Listeria innocua* and *Escherichia coli* determined by WST-8 reduction assay immediately after the treatment and after sample storage for 24 h at 5°C. The data represent mean values and standard deviations of triplicates. Samples: (●) *L. innocua* 0 h, (○) *L. innocua* 24 h, (▲) *E. coli* 0 h, (△) *E. coli* 24 h, (◊) *E. coli*-untreated reference half diluted, (♦) *L. innocua*-untreated reference half diluted.

rupture could therefore not be identified as an immediate effect of the pulsed light treatment.

Active esterase was initially detectable in more than 90% of untreated *L. innocua* (Fig. 3). This percentage did not significantly change up to an applied fluence of 3·24 J cm^{-2}, only at the two highest energy doses, a decrease in CF-positive cells was observed. Similar results were obtained after storage. The staining pattern of *E. coli* with CF was considerably different from *L. innocua* (Fig. 4). In this case, an increase in stained cells occurred up to a fluence of 1·08 J cm^{-2}, which might be attributable to a gradual loss of pump activity as it is known that CF can be actively extruded from some bacteria (Díaz *et al.* 2010). A drop of CF-positive cells occurred similar to *L. innocua* only at high energy levels of 3·24 J cm^{-2} or more. These findings showed that the bactericidal effect of pulsed light is not directly linked to the inactivation of enzymes such as esterases as high percentages of stained cells could still be detected when other viability parameters such as cultivability or metabolic activity were already absent and indicated cell death.

The uptake of $DIBAC_4$, which is indicative for depolarized cells, was detectable immediately after the treatment among a statistically significant fraction of *L. innocua* cells at a fluence of 1·08 J cm^{-2} or higher (Fig. 3). About 10% of untreated *L. innocua* were also depolarized after storage, and this is in line with the above mentioned 10% of cells that showed damaged membranes. An increase to about 20% of stained cells after storage was already detectable at 0·78 J cm^{-2}, so a slight reduction in activity of *L. innocua* overnight could be observed.

With regard to *E. coli*, a statistically significant increase in cells (about 15%) stained with $DIBAC_4$ could be detected at an energy dose of 0·78 J cm^{-2} immediately after the treatment (Fig. 5). After storage, an enhanced depolarization was evident with *E. coli*. About 40% of cells were already depolarized at 0·27 J cm^{-2}. This shows that a progressive loss of vitality occurred during storage in case of *E. coli*. The same trend was observed for the efflux pump activity of *E. coli* (Fig. 5), but the loss of pump activity occurred before the depolarization of the cells, which can be explained by the dependence of efflux activity from the transmembrane electrochemical gradient (Hammes *et al.* 2011). A strong increase in EB-stained cells (more than 70%) was evident even at a relatively low energy dose of 0·27 J cm^{-2} after storage.

Altogether, the flow cytometric data revealed that cellular functions such as esterase activity, membrane integrity, membrane potential or efflux activity were still measurable in a considerable number of bacteria at energy doses that led to an almost complete loss of cultivability. Therefore, it was evident that substantial fractions of both *L. innocua* and *E. coli* populations enter the viable but nonculturable state after a pulsed light treatment. Vitality of *L. innocua* was only slightly further reduced after the samples were stored for 24 h at 5°C, whereas in case of *E. coli*, a clear drop of activity was evident with regard to efflux activity or depolarization of cells. Esterase activity does not seem to be directly affected by pulsed light as well as membrane integrity. In both cases, altered staining pattern was only obvious at high energy doses, and no considerable further change in

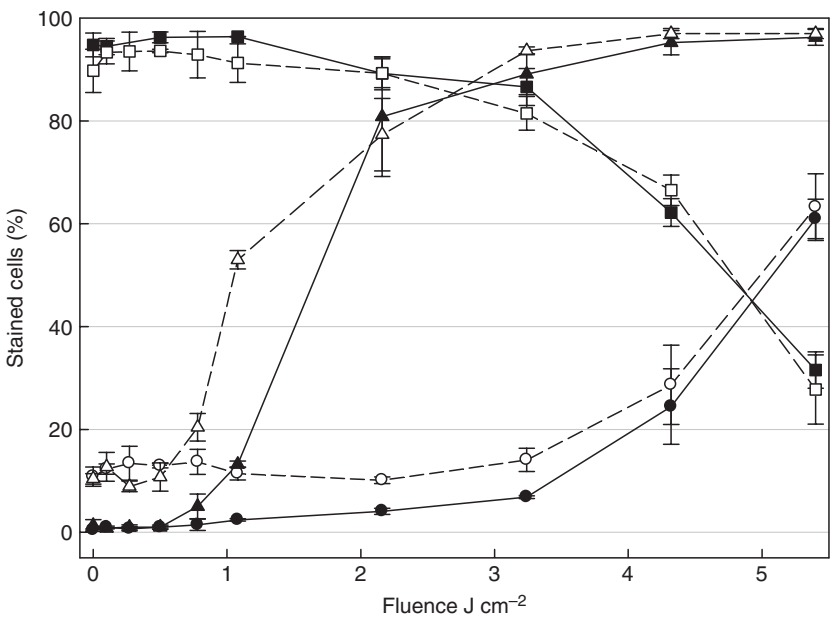

Figure 3 Impact of pulsed light on the physiological state of *Listeria innocua* investigated by fluorescence staining and flow cytometry immediately after the treatment and after sample storage for 24 h at 5°C. The data represent mean values and standard deviations of triplicates. Samples: (●) membrane integrity (PI) 0 h, (○) membrane integrity (PI) 24 h, (■) esterase activity (CFDA) 0 h, (□) esterase activity (CFDA) 24 h, (▲) membrane potential ($DIBAC_4$) 0 h, (△) membrane potential ($DIBAC_4$) 24 h.

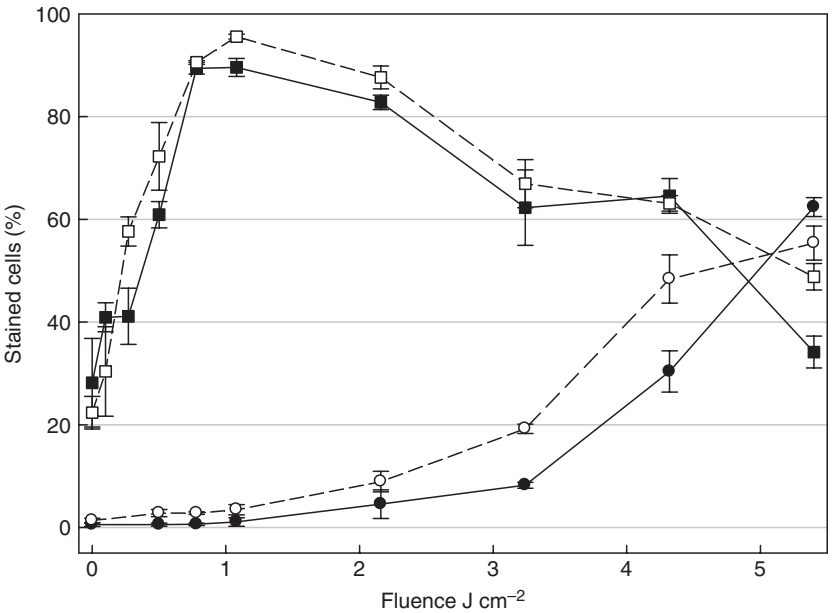

Figure 4 Impact of pulsed light on the physiological state of *Escherichia coli* investigated by fluorescence staining and flow cytometry immediately after the treatment and after sample storage for 24 h at 5°C. The data represent mean values and standard deviations of triplicates. Samples: (●) membrane integrity (PI) 0 h, (○) membrane integrity (PI) 24 h, (■) esterase activity (CFDA) 0 h, (□) esterase activity (CFDA) 24 h.

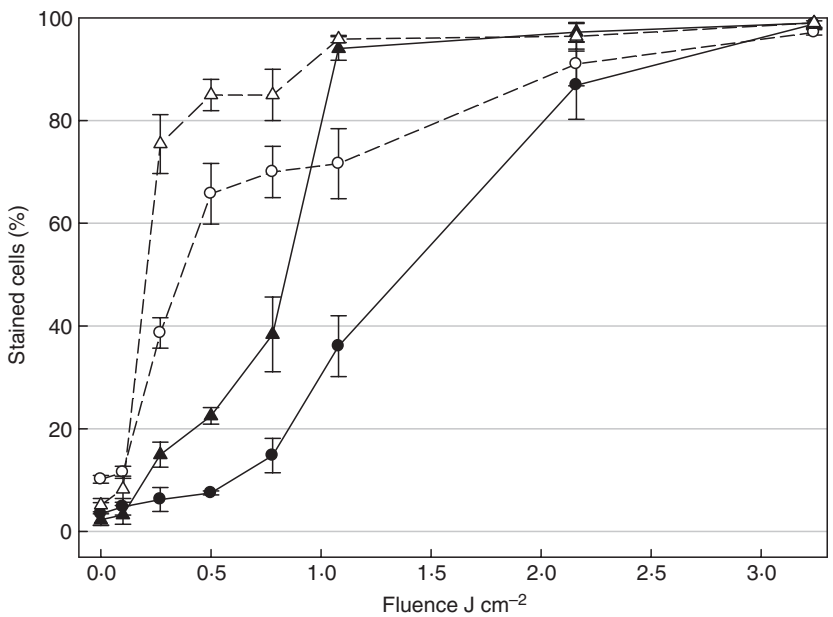

Figure 5 Impact of pulsed light on the physiological state of *Escherichia coli* investigated by fluorescence staining and flow cytometry immediately after the treatment and after sample storage for 24 h at 5°C. The data represent mean values and standard deviations of triplicates. Samples: (▲) efflux activity (EB) 0 h, (△) efflux activity (EB) 24 h, (●) membrane potential ($DIBAC_4$) 0 h, (○) membrane potential ($DIBAC_4$) 24 h.

the staining behaviour was observed for both bacteria after storage.

Detection of reactive oxygen species

The formation of ROS as a consequence of pulsed light treatments was investigated using the oxidative stress-sensitive probe DCFH-DA (Chow *et al.* 2011, Farrell *et al.* 2011). A statistically significant increase in DCF could be observed at a fluence of 0·50 J cm^{-2} for both replicated cultures, which shows that an enhanced formation of reactive oxygen species in *L. innocua* is induced at energy doses where an onset of increasing cultivability loss occurs (Fig. 9).

Detection of DNA damages by RAPD-PCR

Alterations of the banding patterns that were generated from isolated genomic DNA of *L. innocua* after pulsed light treatments by RAPD-PCR with subsequent capillary electrophoresis revealed the occurrence of noticeable DNA damages. A considerable shift in the DNA fingerprints was observable with all 3 primers that were used. The magnitude of alterations in the DNA band patterns

intensified with increasing fluence (Figs 6–8). At 0·10 J cm^{-2}, no significant changes were obvious with all 3 primers, but beginning at a fluence of 0·27 J cm^{-2}, it can be seen from the gel images that the intensities of DNA bands with molecular weights of more than 1000 bp decrease with increasing energy input and in part disappear entirely. The intensity of the band at 1250 bp with primer 2 was considerably reduced at a fluence of 0·27 J cm^{-2} and disappeared completely at 1·08 J cm^{-2} (Fig. 6). A similar progress was noticed with regard to the band at 1900 bp using primer 6 (Fig. 8). The appearance of a weak band with a molecular weight of more than 3000 could be observed with primer 6 for the references at a fluence of 0·10 J cm^{-2}, but this band was already absent at a fluence of 0·27 J cm^{-2}. The amplification of smaller DNA fragments showed in contrast to be much less affected by pulsed light. This is especially evident when the band at 330 bp using primer 5 is considered, where almost no change in band intensity was seen with increasing fluence (Fig. 7). Overall, there is a general trend observable, which indicates that the smaller the DNA fragments, the less affected are their PCR products. On the other hand, it is obvious that several mostly weak bands of lower molecular weight appear with increasing fluence. It is also evident that the overall quantity of DNA that was formed by RAPD-PCR decreases with increasing fluence. At 0·27 J cm^{-2} (Fig. 9), only about 80% of total DNA was formed relative to the untreated controls. This fluence corresponds to the energy dose where an onset of increased cultivability loss occurred.

Discussion

This study has shown that bacterial populations of *L. innocua* and *E. coli* can be inactivated on a heat-stable polysaccharide gel surface by 6 $\log_{10}$ with only one single intense light pulse. Similar efficiencies of pulsed light treatments were demonstrated in different other studies whereby most inactivation tests were performed in suspension or on nutrient agar, with varying instrumental set-up, different equipment and also diverse micro-organisms (Rowan *et al.* 1999; Anderson *et al.* 2000; Gómez-López *et al.* 2005; Wang *et al.* 2005; Farrell *et al.* 2009; Nicorescu *et al.* 2013). Most of these studies report the microbial inactivation efficiency on the basis of colony count reduction tests. In this study, conventional plate count technique revealed a strong and increasing inactivation of both test bacteria with only one single light flash at an energy dose of more than 0·10 J cm^{-2}. The inactivation kinetics exhibited a log-linear trend with an initial plateau at low energy doses, which is most likely due to an injury phase up to a threshold level of energy (Gómez-López *et al.* 2007). No difference in sensitivity between *E. coli* and *L. innocua* was observed by plate count, although other researchers observed a higher resistance of Gram-positive bacteria compared to Gram-negative bacteria (Anderson *et al.* 2000; Farrell *et al.* 2009). However,

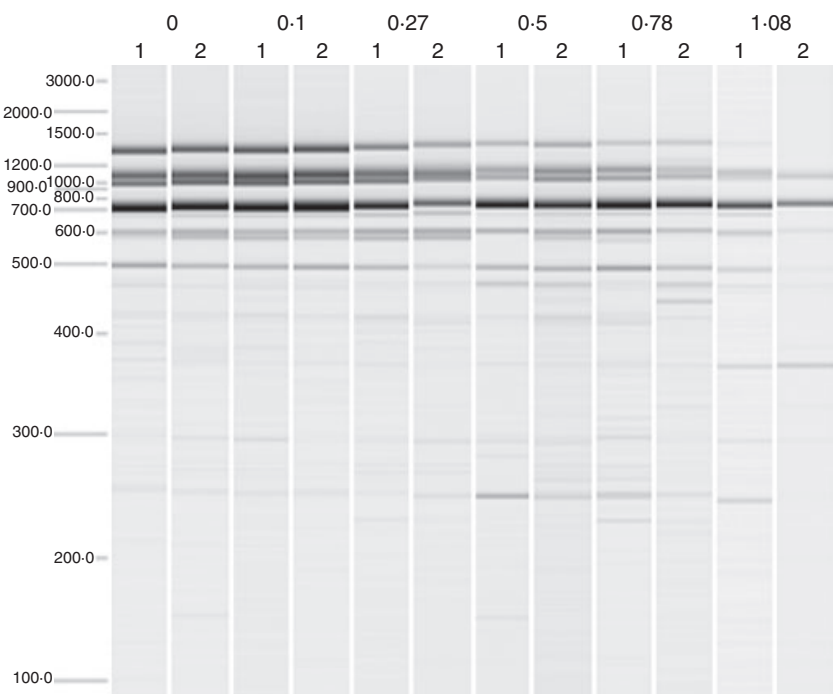

Figure 6 Pulsed light-induced damage to genomic DNA of *Listeria innocua* determined by RAPD-PCR using Primer 2. Applied fluences: 0·10–1·08 J cm^{-2}. Each fluence is represented by two independent replicates 1 and 2.

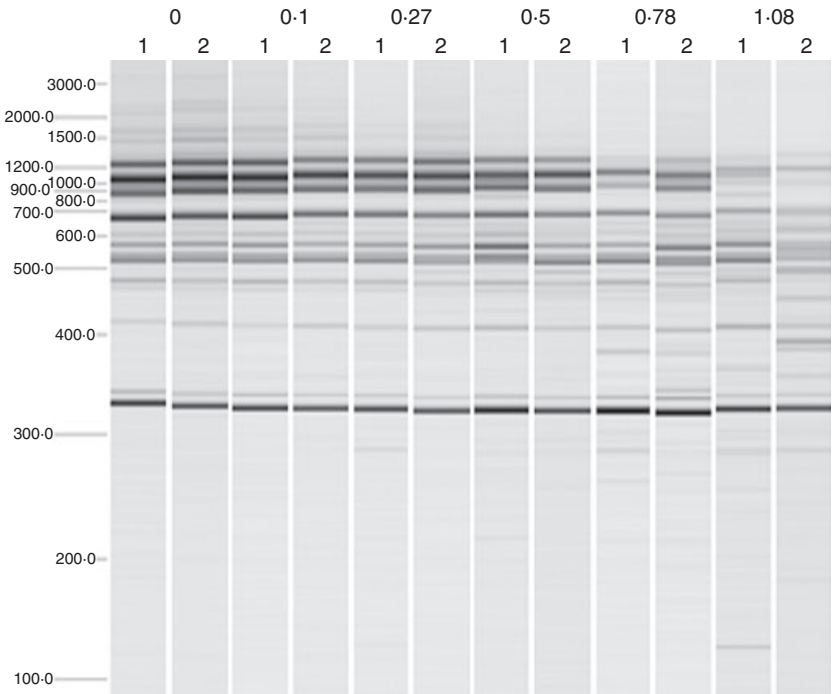

Figure 7 Pulsed light-induced damage to genomic DNA of *Listeria innocua* determined by RAPD-PCR using Primer 5. Applied fluences: 0·10–1·08 J cm^{-2}. Each fluence is represented by two independent replicates 1 and 2.

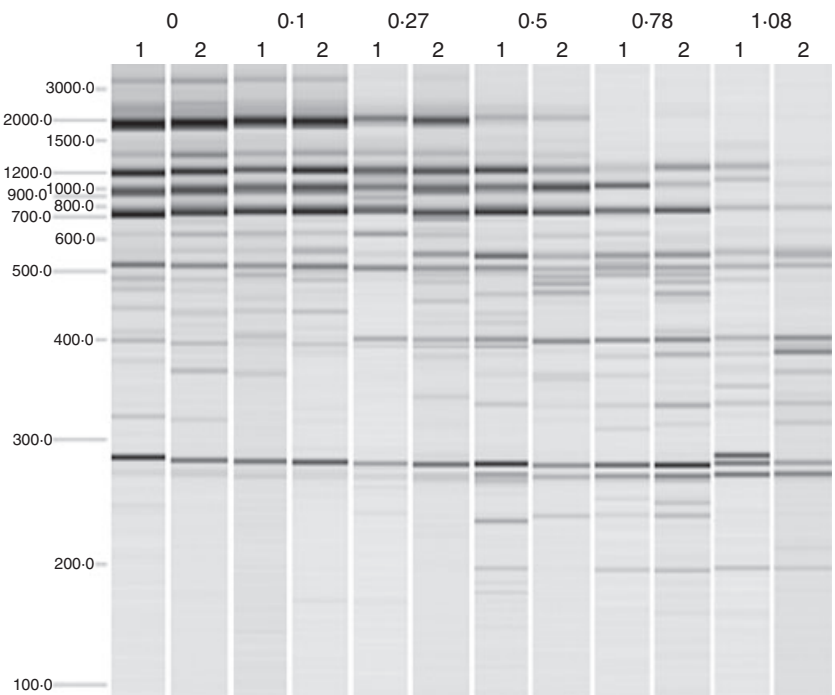

Figure 8 Pulsed light-induced damage to genomic DNA of *Listeria innocua* determined by RAPD-PCR using Primer 6. Applied fluences: 0·10–1·08 J cm^{-2}. Each fluence is represented by two independent replicates 1 and 2.

on the basis of plate counts alone, it is not possible to uncover the impact of such a stress on vital or structural functions of a bacterial cell. Here, it is shown that a more comprehensive image of the cellular response to pulsed light is gained, when multiple methodologies are applied at once. Flow cytometric investigations in combination with different fluorescence probes provide insight into physiological states and are a suitable way to gain further information about the impact of chemical or physical decontamination processes on micro-organisms (Kennedy *et al.* 2011; Nocker *et al.* 2011). Berney *et al.* investigated, for example, the impact of solar disinfection on vital cellular functions in *E. coli* by flow cytometry and concluded that a combination of various culture-independent

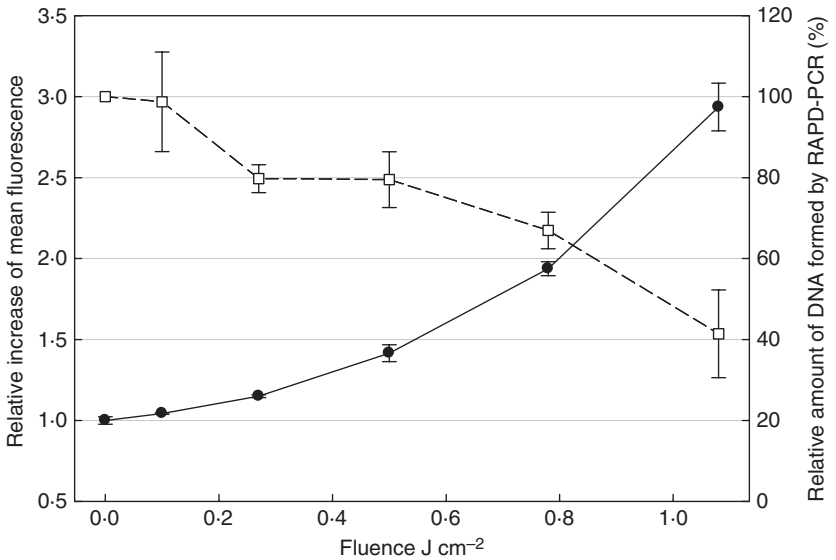

Figure 9 Impact of pulsed light on the amount of amplified genomic DNA of *Listeria innocua* by RAPD-PCR (□) and formation of reactive oxygen species (ROS) (●). Values are normalized to the untreated controls. The data represent mean values and standard deviations of triplicates.

assays gives a detailed picture on the lethal effects of sunlight (Berney *et al.* 2006). Within this work, it was shown that statistically significant levels of metabolic activity of *L. innocua* and *E. coli* can be still detected after a pulsed light treatment even when the colony count dropped already to the detection limit. However, with rising energy input, a gradual shutdown of cellular functions was evident. Similar to the study of Berney *et al.* (2006), active transport systems of stressed cells ceased first, then cytoplasmic membranes depolarized before permeabilization occured (Berney *et al.* 2006). Immediately after applying a fluence of 0·78 J cm^{-2}, high fractions of both bacterial populations were still able to maintain polarized cell membranes, and the level of the metabolic activity was thereby still at more than 30% of the untreated references. At the same time, active esterase was detectable in more than 90% of the cells, and less than 2% showed damaged cell membranes. However, the colony count was already reduced by more than 99·99% in each case. These results show that the inability of bacteria to reproduce on nonselective media does not necessarily mean that the cells are immediately inactive. It is generally accepted that bacteria may still show several vital functions, although they are unable to grow and reproduce in or on nutrient media. This condition is often referred to as viable but noncultivable (VBNC) and can be induced by various chemical or environmental stress factors. Several pathogenic bacteria are known to be able to enter the VBNC state as a survival strategy, among them also *E. coli* and *L. monocytogenes* (Oliver 2010). Flow cytometric analyses can, among other methods, be used as a tool to detect the existence of this special state of bacteria (Khan *et al.* 2010). Wang *et al.* (2010) detected a considerable number of *E. coli* in the VBNC state when the effects of chemically and electrochemically dosed chlorine were assessed by flow cytometry. Numerous other studies have reported that processes that are normally assumed to be bactericidal may result in cells which are in the VBNC state (Oliver 2005). This could lead to erroneous conclusions when evaluating the outcome of inactivation processes because human pathogenic bacteria in the VBNC state may be still infectious or able to retain their virulence after resuscitation (Oliver 2010). Said *et al.* (2012) reported the occurrence of VBNC bacteria after pulsed UV light treatments by investigating phage susceptibilities of *Salmonella typhi*. Infectivity of the host bacteria was still detectable and indicated viability although cultivability was lost (Said *et al.* 2012). Furthermore, Wuytack *et al.* (2003) found that *Salmonella enterica* subjected to pulsed light may result in sublethally injured cells, which was shown by plating on different selective media. Otaki *et al.* (2003) as well as Gómez-López *et al.* (2005) showed the existence of photoreactivation after pulsed light treatments, which is also indicative for the occurrence of the VBNC state (Otaki *et al.* 2003; Gómez-López *et al.* 2005). However, it should also be kept in mind that a lethal stress may lead to cell death in a time-dependent manner, so that vitality functions are still measurable for some time after the cells were subjected to an inactivation process (Hammes *et al.* 2011). In this study, all assays were conducted within 3 h after the treatments and additionally after 24 h storage at 5°C. According to the results, it can be excluded that the pulsed light treatment leads to an immediate shutdown of metabolic activity, inactivation of intracellular esterase or membrane disruption. *L. innocua* was furthermore able to maintain their vitality functions almost at the same level after storage for 24 h at 5°C, whereas the

energetic state of *E. coli* was distinctively reduced after storage. It is therefore likely that considerable time-dependent differences in the ability of bacteria to maintain certain energy levels after a pulsed light treatment exist, although identical inactivation kinetics by plate count are obtained. Bosshard *et al.* (2009) also observed a continued deterioration of the physiological state of *Salmonella typhimurium* and *Shigella flexneri* after solar disinfection and subsequent dark storage so apparently the cells were unable to repair damage caused by radiation. Similar to the present study, no recovery of irradiated bacteria was evident (Bosshard *et al.* 2009).

The measurement of intracellular esterase activity proved to be a weak parameter to investigate cell viability because high levels of CF-stained bacteria could be detected even when cells were already nonculturable and de-energized. The detection of enzyme activity does therefore not necessarily suggest cell viability (Hammes *et al.* 2011). The exclusion of PI is often taken as criteria for live bacteria, but in this study, this parameter could also not be seen as a suitable marker for viability because high levels of cells with intact membranes were detected after treatment at lethal energy doses, the interpretation of membrane integrity analysis should therefore be taken with care (Hammes *et al.* 2011). Similar results were obtained by Schenk *et al.* (2011) who investigated the effect of a UV-C treatment on *L. innocua* by flow cytometry using CFDA and PI in a double staining assay. They observed that after a treatment for 2 min, only 9·4% of the detected cells showed damaged membranes and absent metabolic activity although the plate count was reduced by 3·6 $\log_{10}$. Therefore, it was evident that the inactivation mechanism of *L. innocua* by UV-C is not primarily related to membrane damages or inactivation of intracellular enzymes such as esterases, but predominantly caused by the breakdown of the DNA molecules (Schenk *et al.* 2011). Fröhling *et al.* (2012) investigated the effect of an atmospheric pressure plasma treatment on *L. innocua* and *E. coli* by flow cytometry using also CFDA and PI. The treatment was conducted on the same gel carrier that was applied in this study. They observed that the permeability of *L. innocua* for PI was only slightly increased with increasing treatment time and concluded that this may be explained by the higher strength and rigidity of Gram-positive bacteria. Like in the present study, cell lysis of *L. innocua* occurred only after high operating powers, so that it seemed most likely that RNA and DNA were directly affected by the plasma treatment (Fröhling *et al.* 2012). Ferrario *et al.* (2013) analysed pulsed light-induced damages on *Saccharomyces cerevisiae* in peptone water by flow cytometry using PI and CFDA as well. Similar to the present study, they observed the occurrence of stressed cells that lost their ability to grow in agar but still showed metabolic activity. They also stated that much more log reductions in viable cells by the CFU method were obtained in comparison with the log increase in permeabilized cells (Ferrario *et al.*2013).

It is generally assumed that the UV region is the most important part in the spectrum of pulsed light (Gómez-López *et al.* 2007), causing a variety of mutagenic and cytotoxic DNA lesions (Rastogi *et al.* 2010) that lead to microbial inactivation. Wang *et al.* (2005) showed that the maximum inactivation of *E. coli* by pulsed light is obtained at 270 nm, a wavelength which is highly absorbed by DNA. Rowan *et al.* (1999) found that light pulses of high UV content are more efficient than flashes with a low UV content for the inactivation of food-related micro-organisms, and Woodling and Moraru (2007) observed that no inactivation of *L. innocua* by pulsed light can be obtained when cells are exposed only to visible or near-infrared light (Woodling and Moraru 2007). However, there is evidence that not only photochemical effects due to UV light are responsible for the bactericidal action of pulsed light. It is believed that besides photochemical effects, also photothermal or photophysical mechanisms may play some role in the lethal action of pulsed light and that microbial inactivation is not equivalent to continuous UV irradiation (Wekhof 2000; Elmnasser *et al.* 2007; Gómez-López *et al.* 2007; Oms-oliu *et al.* 2010; Farrell *et al.* 2011). Several authors have reported the rupture of microbial cells by pulsed light as an additional inactivation mechanism. Microscopic observations by Krishnamurthy *et al.* (2008) indicated severe cellular damages with *Staphylococcus aureus* after a 5-s pulsed UV light treatment, although no temperature increase was measurable. The cell wall damage, cytoplasmic membrane shrinkage, cell content leakage and mesosome rupture were therefore attributed to the pulsing effect (Krishnamurthy *et al.* 2008). Takeshita *et al.* (2003) investigated the effect of pulsed light on yeast cells and compared it to continuous UV light. They found distinct membrane damages of yeast cells only when the cells were treated with pulsed light, but on the other hand, slightly higher DNA damage with continuous UV light. It was therefore concluded that differences in the mode of inactivation between UV light and pulsed light are existent (Takeshita *et al.* 2003). Wekhof (2000) proposed that a momentous overheating of cellular constituents by the absorption of all UV light from a flash lamp might lead to the vapourization of water and result in the disruption of bacterial cells (Wekhof 2000). However, in this study, membrane damages of *L. innocua* and *E. coli* were only detected far beyond cultivability loss at fluencies of 3·24 J cm^{-2} or higher and also not among the whole populations. Therefore, in this case, membrane disruption due to a temporary overheating does not seem

to be a primary cause of microbial inactivation. Enzymes such as esterases are in general heat sensitive, this was observed with the heat-treated negative controls of *L. innocua* and *E. coli* (85°C, 10 min), where active esterase was completely absent. In the present study, a decrease in esterase activity could also only be observed at high fluencies of more than 3·24 J cm^{-2}, which shows that there is no direct correlation between the loss of cultivability and an inactivation of those enzymes. This decrease was furthermore accompanied by an increased uptake of PI, so it might also be possible that the drop in esterase activity at high fluencies is attributable to the occurrence of compromised membranes. A decrease in fluorescent bacteria may therefore be a result of CF leakage from the cells and not caused by inactivated enzymes due to pulsed light (Tracy *et al.* 2010). The observed low percentage of CF-stained *E. coli* cells at low fluencies is most likely attributable to active efflux of this dye and is therefore also indicative for vitality (Díaz *et al.* 2010). Anderson *et al.* (2000) stated that the inactivation of micro-organism could not be attributed to a photothermal action, as temperature rise in the treated agar was negligible (Anderson *et al.* 2000). Nicorescu *et al.* (2013) did not observe any disruption of *Bacillus subtilis* when the cells were exposed to pulsed light in suspension, but SEM images revealed damaged cells when they were inoculated on different spices. They concluded that different inactivation mechanisms may be involved when pulsed light is applied in dry state or in liquid (Nicorescu *et al.* 2013). Levy *et al.* (2012) did not observe any structural alterations of *B. subtilis* or *Aspergillus niger* spores when exposed to pulsed light on polystyrene (Levy *et al.* 2012). Based on transmission electron micrographs of pulsed light-treated *S. cerevisiae* cells, Ferrario *et al.* (2013) stated that various cell structures other than plasma membranes were altered and/or destroyed in a different degree depending on exposure time and type of medium (Ferrario *et al.* 2013). It has furthermore been reported that micro-organisms differ in their sensitivity to pulsed light, and this may be related to their cell wall composition as well as to their protective and repair mechanisms (Anderson *et al.* 2000; Oms-oliu *et al.* 2010). Endospores and conidiospores have in general shown to be more resistant to pulsed light than vegetative cells (Anderson *et al.* 2000). Due to these variable results that were obtained in different studies concerning a potential rupture of micro-organisms by high-intensity light flashes, it seems likely that the occurrence of photothermal or photophysical inactivation mechanisms is to some extent dependent on the characteristics of the particular micro-organisms such as their size, cellular structure and UV light absorption properties. Among different studies, the cells were either treated in suspension on nutrient agar or on dry surfaces so that the particular matrix in which the micro-organisms are embedded could also have an impact on the magnitude of occurring cellular damage. The used equipment and experimental set-up may also interfere with the outcome of such investigations, because one unique energy dose can be applied by continuous light, many light flashes or only one single light flash. This has raised the debate about a possible peak power dependence of microbial inactivation efficiency (Gómez-López *et al.* 2007), which needs further investigations.

To elucidate possible photochemical effects of pulsed light and to detect cellular damages that directly account for the loss of cultivability of *L. innocua*, the intracellular formation of reactive oxygen species (ROS) was investigated with the ROS-sensitive probe DCFH-DA, and the occurrence of possible DNA damages was investigated by RAPD-PCR. It is well known that UV light induces the formation of ROS in bacteria, which can cause oxidative damages of lipids, proteins and DNA (Kumar *et al.* 2004; Rastogi *et al.* 2010, 2011; Santos *et al.* 2012, 2013). Intense pulsed light flashes include all UV regions, and it was reported that microbial inactivation efficiency is highest with UV-C and lowest with UV-A radiation (Santos *et al.* 2013). UV-A is known to enhance the production of ROS, whereas UV-B and UV-C photons also cause direct DNA damages by inducing the formation of photoproducts causing adverse effects of the genome stability (Santos *et al.* 2013). The formation of mutagenic and cytotoxic DNA lesions such as cyclobutane-pyrimidine dimers (CPDs), 6–4 photoproducts (6–4 PPs) and their Dewar valence isomers as well as DNA single- or double-strand breaks is induced. These lesions can block the movement of DNA polymerase on DNA template and hamper DNA replication and transcription which may ultimately lead to cell death. To cope with such damages, organisms have developed a number of repair mechanisms such as photoreactivation, nucleotide excision repair, base excision repair or mismatch repair (Kumari *et al.* 2008; Rastogi *et al.* 2010, 2011; Beauchamp and Lacroix 2012). Statistically significant levels of ROS were detected at 0·50 J cm^{-2}, which corresponds to an energy dose where an increasing loss of cultivability occurred. This indicates that oxidative stress is directly linked to the killing effect of pulsed light. Farrell *et al.* (2011) also investigated the formation of ROS after treating *Candida albicans* with pulsed light and furthermore detected DNA damages using comet assay (Farrell *et al.* 2011). Damage to genomic DNA of *L. innocua* due to pulsed light could be detected in this study by RAPD-PCR as well. This technique has been successfully employed in various studies to investigate DNA damages in bacteria caused by genotoxic agents or radiation (Atienzar *et al.* 2002; Kumar *et al.* 2004; Muranyi *et al.*

2010; Rastogi *et al.* 2011). It is based on the amplification of genomic DNA with single primers of arbitrary nucleotide sequence which anneal to multiple regions of the DNA. The PCR generates a number of amplicons of variable length which can be separated by electrophoresis to generate a DNA fingerprint (Atienzar *et al.* 2002). Alterations in the band pattern account for DNA adducts, breaks and mutations, and this may lead to decreased intensities, complete loss or even to appearance of new bands when genetic rearrangements take place that create new annealing sites for oligonucleotide primers (Atienzar and Jha 2006). Amplicons of higher molecular weight are usually more affected than smaller segments as it is statistically more likely that damage occurs between two primers that encompass longer DNA sequences (Atienzar *et al.* 2002; Muranyi *et al.* 2010). This tendency was observed with all three primers that were used in this study. A pronounced disappearance of bands of higher molecular weight was also observed by Kumar *et al.* (2004) who investigated UV-B-induced DNA damages in Cyanobacteria. Furthermore, they also found a distinctive DNA band of 400 bp that was almost not affected and concluded that this might be attributable to DNA regions that are resistant to UV-B radiation (Kumar *et al.* 2004). The appearance of such a band was also found in this study with primer 5, where the intensity of the band at 330 bp was not affected irrespective of the applied fluence. Overall, noticeable alterations in the band patterns occurred beginning at 0·27 J cm^{-2}, so it can be concluded that the loss of cultivability was directly linked to the formation of ROS and DNA damages. However, it remains unclear, which kind of damage is actually responsible for the changes of the band patterns. It is most likely that different photoproducts such as CPDs and 6–4 PPs are formed because these types of lesions are commonly found as a result of UV light (Beauchamp and Lacroix 2012). Cheigh *et al.* (2012) reported the occurrence of double-strand breaks, single-strand breaks and CPDs with a similar pattern when *E. coli* and *L. monocytogenes* were either treated with pulsed light or continuous UV-C (Cheigh *et al.* 2012). Besides damages to DNA, microbial inactivation by pulsed light could also be linked to alterations of proteins and lipids. The occurrence of lipid peroxides and carbonylated proteins can be a consequence of UV light disinfection (Santos *et al.* 2012, 2013), and lipid hydroperoxidation was found in the cell membrane of pulsed light-treated yeast (Farrell *et al.* 2011). However, the contribution of these kinds of cellular lesions on bacterial inactivation by pulsed light has not been shown yet.

In this study, it was shown that pulsed light has proven to be an efficient way to inactivate *L. innocua* and *E. coli* on the surface of a heat-stable polysaccharide gel. However, differing results were obtained when the inactivation efficiency was evaluated by traditional plate count technique or on the basis of various viability staining approaches. Considerable percentages of bacterial populations still showed active esterases, polarized membranes, metabolic activity and intact membranes at fluencies where cultivability was already completely lost. A higher energy dose is required to induce an immediate and complete shutdown of cellular vital functions and to prevent the occurrence of viable but noncultivable bacteria. The loss of reproducibility of *L. innocua* was directly accompanied by the formation of intracellular ROS and damages to DNA. The inactivation process of bacteria by intense light pulses seems in this case primarily to be attributable to photochemical rather than photothermal or photophysical effects. Further research is, however, needed to elucidate the impact of pulsed light on other kinds of food spoilage micro-organisms such as yeast or moulds, to identify further possible cellular lesions that lead to microbial inactivation and to investigate the response of micro-organisms to pulsed light on foodstuff.

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Conflict of interest

No conflict of interest declared.

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