Journal of Microbiological Methods 184 (2021) 106208

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Production of inactivated gram-positive and gram-negative species with preserved cellular morphology and integrity

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ARTICLE INFO

Keywords:
Bacterial inactivation
Standardized protocol
Beta-propiolactone
Pasteurization
Heat-treatment
Scanning electron microscopy
Bacterial ghosts

ABSTRACT

There are many approaches available to produce inactive bacteria by termination of growth, each with a different efficacy, impact on cell integrity, and potential for application in standardized inactivation protocols. The aim of this study was to compare these approaches and develop a standardized protocol for generation of inactivated Gram-positive and Gram-negative bacteria, yielding cells that are metabolically dead with retained cellular integrity i.e., preserving the surface and limited leakage of intracellular proteins and DNA. These inactivated bacteria are required for various applications, for instance, when investigating receptor-triggered signaling or bacterial contact-dependent analysis of cell lines requiring long incubation times. We inactivated eight different bacterial strains of different species by treatment with beta-propiolactone, ethanol, formalin, sodium hydroxide, and pasteurization. Inactivation efficacy was determined by culturing, and cell wall integrity assessed by quantifying released DNA, bacterial membrane and intracellular DNA staining, and visualization by scanning electron microscopy. Based on these results, we discuss the bacterial inactivation methods, and their advantages and disadvantages to study host-microbe interactions with inactivated bacteria.

1. Introduction

Bacterial inactivation refers to methods that result in termination of bacterial growth by damaging DNA or protein synthesis, often resulting in impaired cellular integrity. The aim of this study is to evaluate the protocols for the efficacy of bacterial inactivation while preserving the surface structure with minimal leakage of intracellular proteins and DNA for their potential to use them for standardized inactivation. While several effective techniques are available to achieve bacterial inactivation(Goncalves et al., 2014; Kniggendorf et al., 2011; Langemann et al., 2010; Parker et al., 1950), most studies focus on single bacteria and it is mostly unknown whether the bacterial surfaces are preserved for both Gram-positive and Gram-negative bacteria. For standardized inactivation of multiple bacteria in a complex mixture such as a fecal sample or when investigating many different bacteria at the same time a general method is preferred (Taddese et al., 2020).

Commonly applied bacterial inactivation methods are antibiotics and bacterial cell lysis but these are accompanied with particular limitations(McDonnell and Russell, 1999; Russell, 1999). When performing a standardized inactivation of various bacteria i.e., multiple bacteria are inactivated in a consistent manner, then antibiotic treatments are impractical due to antibiotic resistance, especially when dealing with clinical strains (Levy and Marshall, 2004). Noticed from practical experience, receptor-triggered signaling or bacterial contact-dependent analysis often necessitates long incubation times (6 h to days) requiring frequent media refreshments, antibiotic elimination after a few hours or use of inactivated bacteria (Boleij et al., 2011; Pleguezuelos-Manzano et al., 2020). For instance, when investigating effects of bacteria on cell proliferation incubations from 24 to 72 h are needed that are impossible with live bacterial cells (Taddese et al., 2020). Methods resulting in

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https://doi.org/10.1016/j.mimet.2021.106208
Received 7 January 2021; Received in revised form 26 February 2021; Accepted 19 March 2021
Available online 22 March 2021

porous membranes are disadvantageous since intracellular products leak out and may affect analysis (Pillet et al., 2016; Yao et al., 2014); i.e. whether the observed effect results from bacterial intracellular products or extracellular membrane proteins. Recently, bacterial ghosts emerged due to their preserved structure. Bacterial ghosts are inactivated bacteria whose cytoplasmic content including DNA have escaped via perforated membranes (Langemann et al., 2010; Vinod et al., 2014; Wu et al., 2017). They are applied for creation of vaccines to immunize humans and animals, for analysis of cell response to bacterial outer-membrane structures, and used to inactivate bacteria as carriers for drugs or antigens (Langemann et al., 2010). Nevertheless, these also come with certain challenges that our study is aiming to solve in addition to the challenges that arise when implementing a standardized bacterial inactivation protocol.

One challenge for a standardized protocol for bacterial inactivation lies in the diversity of the targeted bacteria. For example, the structurally different walls of Gram-positive and Gram-negative bacteria may inhibit certain inactivation treatments. One example is the production of bacterial ghosts by using a plasmid with an E gene insert derived from bacteriophage ΦX174, that lyses Gram-negative bacteria by forming a lysis tunnel across the double membrane, where the DNA and cytoplasmic contents escape (Halfmann et al., 1993; Langemann et al., 2010). Additionally, DNA is then degraded by beta-propiolactone (BPL) and/or staphylococcal nuclease A (Haidinger et al., 2003; Perrin and Morgeaux, 1995). Finally, inactivated bacteria are lyophilized to ensure inactivation. This procedure inactivates a range of Gram-negative bacteria as their double membrane (cytoplasmic and outer membrane) is necessary for the formation of a lysis tunnel, but it is not compatible with Gram-positive bacteria due to the lack of an outer membrane (Halfmann et al., 1993; Langemann et al., 2010).

A second challenge lies in finding inactivation methods for application in standardized inactivation protocols suitable for Gram-positive as well as for Gram-negative bacteria. Gram-positive bacterial ghosts were created for *Listeria monocytogenes* (referred to as *L. monocytogenes* ghosts or LMGs) using a combination of chemicals in a series of steps (Wu et al., 2017). With these chemicals, the minimum inhibitory concentrations had to be determined beforehand to produce inactivated bacteria with preserved structure. The minimum inhibitory concentrations may differ for individual bacteria (Park et al., 2016), making this inactivation treatment unsuitable for standardized protocols when investigating several different bacteria simultaneously.

A third challenge when standardizing the inactivation protocol lies in the methods to quantify bacterial cells. There are numerous methods to quantify bacteria with different accuracy and different suitability for application in high-throughput, including plating and counting colony forming units (CFU), 4,6-diamidino-2-phenylindole (DAPI)-staining, fluorescent-activated cell sorting (FACS), and measuring optical density (OD) (Davis, 2014; Seo et al., 2010). DAPI and FACS require further processing of bacteria which end up being time-consuming when inactivating many different bacteria simultaneously. Measuring OD may be quicker and easier, albeit less accurate than plating and counting CFUs (Francois et al., 2005). Since CFU counting requires overnight incubations of bacteria on agar plates (depending on the bacterial species), OD may be measured in parallel to enable the continuation with experiments immediately.

Our study aims to solve the limitations of antibiotic treatments (resistance), bacterial cell lysis (release of intracellular proteins), and "bacterial ghosts" (inactivation of only Gram-negative bacteria) when studying complex bacterial mixtures or analyzing multiple bacterial species simultaneously. We aimed at finding inactivation methods that create intact inactivated bacteria whose DNA remained within the cell, as measure of cell integrity. Inactivation may include membrane leakage of low-molecular weight cellular contents such as potassium (Huffer et al., 2011; Yao et al., 2014). We compared five protocols for their ability to effectively inactivate bacteria while preserving the bacterial membrane structure, applicability to different bacterial strains of different species, and the possibility for standardization, including four chemical treatments beta-propiolactone (BPL), ethanol, formaldehyde (further referred to as formalin), sodium hydroxide (NaOH), and two physical treatments i.e., pasteurization and ultraviolet (UV-C) light.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Eight strains of bacteria from five phyla were selected as representatives for the structural differences in bacterial cell walls of Gram-positive and Gram-negative strains to evaluate the inactivation efficacy of each method (Table 1). The anaerobic bacteria *Bacteroides fragilis* 9343 NTBF, *Parabacteroides distasonis* 3999B T(B)4, *Fusobacterium nucleatum* patient isolate NTB17 (from the Radboudumc strain collection), *Akkermansia muciniphila* ATCC BAA-835 were cultured in an anaerobic jar using sachets (Thermo Fisher Scientific, USA) in Brain-Heart-Infusion (BHI) broth (Sigma-Aldrich, USA) supplemented with L-cysteine (Sigma-Aldrich, USA), yeast extract (BD, USA), hemin (Sigma-Aldrich, USA), vitamin K_1 (Sigma-Aldrich, USA) for 48 h at 37 °C. Facultative aerobic strains such as *Salmonella enterica* serovar Typhimurium NTB6 (Kortman et al., 2014) (further designated as *S. typhimurium*), *Streptococcus gallolyticus* subsp. *gallolyticus* UCN34 (further designated as *S. gallolyticus*), *Escherichia coli* NC101 and *Lactococcus lactis* IL1403 were cultured aerobically in BHI broth overnight at 37 °C with 5% CO_2.

Following incubation, optical density at 620 nm was measured in the microplate reader Infinite F50 (Tecan, Switzerland) and samples were centrifuged at maximum speed (16,100 x g) for 10 min. Supernatants were discarded and OD_{620} was adjusted to 1.0 in 0.9% sodium chloride buffer (B Braun Melsungen AG, Germany) for treatments with BPL (Acros Organics, Thermo Fisher Scientific, USA) and pasteurization. For NaOH, ethanol (all from Merck, Germany) and formalin (formaldehyde solution about 37%, Merck, Germany) treatments, pellets were resuspended at OD_{620} of 1.0 in NaOH, ethanol and formalin, respectively.

2.2. Inactivation treatments

2.2.1. BPL treatment

Bacteria were dissolved in 0.9% sodium chloride solution. BPL was added to sodium chloride solution at concentration of 1:2000 (v/v) and samples were incubated rotating at 150 rpm at 4 °C overnight (modified protocol from *Gonçalves* et al. (Goncalves et al., 2014)). After incubation, samples were placed at 37 °C for 2 h to inactivate BPL. No rinsing was required.

Table 1
Strains, their phyla and properties.

Bacteria	Phyla	Oxygen requirements	Gram staining reaction
Akkermansia muciniphila	Verrucomicrobia	Anaerobe	Negative
Bacteroides fragilis	Bacteroidetes	Anaerobe	Negative
Fusobacterium nucleatum	Fusobacteria	Anaerobe	Negative
Parabacteroides distasonis	Bacteroidetes	Anaerobe	Negative
Escherichia coli	Proteobacteria	Facultative aerobe	Negative
Lactococcus lactis	Firmicutes	Facultative aerobe	Positive
Streptococcus gallolyticus	Firmicutes	Facultative aerobe	Positive
Salmonella typhimurium	Proteobacteria	Facultative aerobe	Negative

2.2.2. NaOH, ethanol and formalin treatments

Bacterial pellets (Table 1) were resuspended in 6 mg/ml (0.15 M) NaOH solution (Rabi et al., 2018; Vinod et al., 2014), 70% ethanol (Morton, 1950), or formalin (McDonnell and Russell, 1999), and incubated at room temperature for 5 min.

2.2.3. Pasteurization

Bacteria were heat-treated in a dry block heater (Grant, UK) at 70 °C for 30 min (Coleman et al., 2007; Zhang et al., 2010). Hereafter, samples were immediately placed on ice.

2.2.4. UV-C light

Bacteria in PBS were placed under a UV-C light in a flow-cabinet without plastic lid overnight. Hereafter, cells were resuspended in PBS (due to evaporation of the liquid), centrifuged at 16.100 ×*g* and the pellet was resuspended in PBS.

Immediately after inactivation, 10-fold serial dilutions were obtained in PBS for CFU determination (see below). The remaining inactivated bacteriawere centrifuged at16,100 ×*g*, for 10 min, where the top part of the supernatants were transferred into a new microcentrifuge tube to measure the DNA concentration. Using 0.9% sodium chloride, the bacterial pellets were resuspended for rinsing and centrifugation twice.

2.3. Colony forming unit (CFU) determination

Following inactivation treatments, 10-fold serial dilutions were plated with treated and untreated bacteria to determine the CFU and compare the inactivation efficacy. Three drops (15 µl each) from each dilution were placed on BHI agar plates and incubated aerobically (37 °C, 5% CO_2, overnight) and anaerobically (37 °C, 48 h) for facultative aerobes and anaerobes, respectively (Boleij et al., 2011). After incubation, colonies were counted and CFU/ml were calculated for each inactivation method. Hereby, the number of bacteria prior to inactivation treatments at OD_{620} of 1 were determined.

2.4. Measurement of the concentration of extracellular DNA

After inactivation treatments, bacteria were centrifuged at 16,100 ×*g* for 10 min and DNA concentration was measured from the top fraction of the supernatants at 260 nm/280 nm ratio using NanoDrop ND-1000 (Isolagen Technologies, USA).

2.5. Fluorescein isothiocyanate (FITC) labeling and 4′,6-diamidino-2-phenylindole (DAPI) staining

2.5.1. FITC labeling and DAPI staining of live bacteria

Bacteria were grown overnight and OD_{620} was adjusted to 1.0. Bacteria were washed once with phosphate buffered saline solution (PBS) and centrifuged at 16,100 ×*g* for 3 min. During centrifugation, FITC (Sigma-Aldrich, USA) was dissolved in dimethyl sulfoxide (DMSO, PanReac AppliChem) at 5 mg/ml and diluted in PBS at 0.5 mg/ml (FITC/PBS mixture). For each bacterium negative controls were live bacteria, stained with FITC but not DAPI (Prolong^{Tm} Gold Antifade Mountant with DAPI, Thermo Fisher Scientific, USA) (Fig. 1). Positive controls are stained with both, DAPI and FITC. Following centrifugation, bacterial pellets and positive controls were resuspended in 1 ml FITC/PBS mixture. Bacteria were incubated rotating in the dark for 30 min for labeling. Afterwards, they were washed with PBS 3 times to remove non-bound FITC (centrifugations at 16,100 x g for 3 min).

2.5.2. Inactivation treatments

Except for positive and negative controls, bacteria were inactivated with the above described treatments.

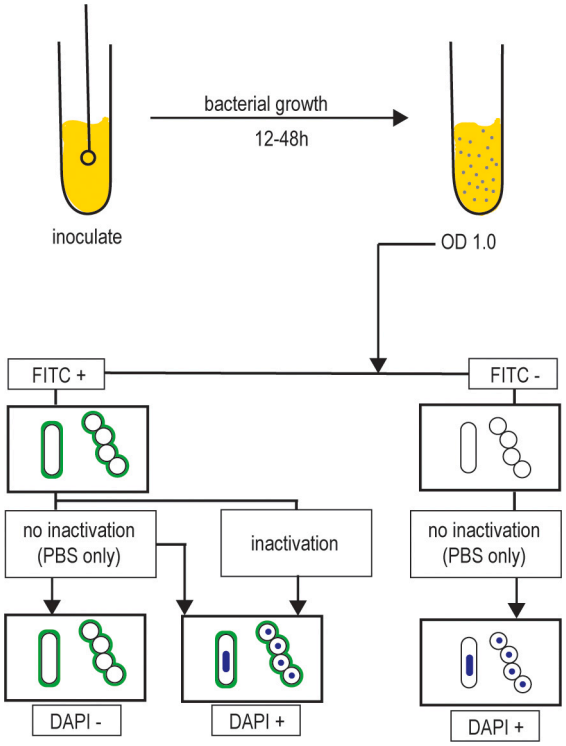

Fig. 1. Overview of FITC/DAPI labeling. First bacteria were grown at their specific conditions (see methods) to stationary phase and adjusted to an OD of 1.0 in PBS. Bacterial cells were either labeled with FITC in PBS or alternatively incubated in PBS only (non-labeled control). Next, FITC-labeled bacteria were inactivated with the respective inactivation treatments 70% ethanol, formalin, NaOH 6 mg/ml, BPL 1:2000 (v/v) or pasteurization at 70 °C. After inactivation inactivated FITC labeled bacteria and non-inactivated FITC labeled and non-labeled bacteria were stained with DAPI (non-inactivated FITC/DAPI-labeled control and non-labeled DAPI-only control). A control of FITC-labeled non-inactivated bacteria without DAPI staining was used as FITC-only control.

2.5.3. Fixation

Inactivated cells were centrifuged at 16,100 ×*g* for 3 min and pellets were resuspended in formalin for 5 min. Formalin was washed out with PBS 3 times (centrifugations at 16,100 ×*g* for 3 min). Negative and positive controls of each bacterium that were not fixed and permeabilized, interfered with DAPI-staining, but there was no difference in FITC staining between these controls and inactivated bacteria (results not shown), therefore, formalin-fixed/inactivated bacteria served as reference for fluorescence microscopy.

2.5.4. Staining with DAPI and preparation for microscopy

After washing out formalin, bacteria were resuspended in 1 ml PBS which correlates to 1 OD (adjusted in the beginning; Table S1). Using the bacteria at 1 OD, dilutions of 10^1 or 10^2 were performed of which 5 µl of bacteria were placed on slides. Bacteria were air dried on the slides and then covered with 1–2 drops of Prolong^{Tm} Gold Antifade Mountant with DAPI (Invitrogen) and cover slips. For negative controls, Quick-D mounting medium (Klinipath, The Netherlands) was used. The slides were dried overnight and subsequently stored at 4 °C in the dark until imaging for a maximum of 3 months. Finally, bacteria were visualized under the fluorescence microscope (Leica DMRA) at 1000× magnification (with oil). Pictures were made using Leica AF software and the Leica DFC420 camera (Leica).

2.6. Scanning electron microscopy (SEM)

2.6.1. Fixation

Following DNA measurements, remaining supernatants were

completely removed and bacterial pellets were fixed in 2% glutaraldehyde diluted in 0.1 M cacodylate buffer overnight at 4 °C. Samples were centrifuged, washed with 0.1 M cacodylate buffer and stored at 4 °C in same buffer until use. When ready for SEM visualization, post-fixation was performed. For this, buffer was removed by centrifugation and bacteria were incubated in 1% osmium tetroxide for 1 h at room temperature. Samples were centrifuged and washed with deionised water.

2.6.2. Dehydration

Bacteria were placed on filter papers with pore size of 5 to 13 μm (Thermo Fisher Scientific, USA) that were cut to about 12 mm in diameter and hydrated with few drops of water. Vacuum-suction was applied for bacteria to stick on the filters. Dehydration was performed in ethanol series of 50%, 70%, 80%, 96% and 100%. Filters must be kept wet at all times, so some ethanol was always left at the bottom every time the solution was exchanged.

2.6.3. Drying

Hexamethyldisilazane (HMDS, Sigma-Aldrich, USA) was used for drying bacterial samples. For drying the filters, three HMDS dilutions (2:1, 1:1 and 1:2) were prepared using 100% ethanol. Filters must be kept wet by leaving some solution during exchange.

2.6.4. Coating

Filters were attached to aluminum Zeiss pin stubs with 12.7 mm diameter (MicrotoNano, The Netherlands) and coated with gold in HHV Scancoat Six bench-top sputter coater (HHV Ltd., UK) 3 times for 30 s each.

2.6.5. SEM visualization

Preservation and damage of bacterial surface structures of the bacteria after inactivation treatments were compared to that of untreated bacteria at comparable magnifications and settings using Zeiss Sigma 300 (Carl Zeiss, Germany).

2.7. Quantification of dents and extracellular structures (ECS) in SEM pictures

Dents, defined as a slight hollow in the bacterial cell surface, and ECS, defined as small bulges which appear on the surfaces, are observed on bacterial surfaces in SEM pictures (Hartmann et al., 2010). For quantification, pictures with fewer than 50 bacteria were selected to be able to judge individual bacterial cells. The number of dents and ECS on each cell were counted and the percentages of total dents or ECS were calculated.

2.8. Statistics and reproducibility

All experiments were performed in duplicate and repeated at least once in an independent experiment. For the analysis of released DNA (*eDNA*) treatments of all bacteria combined ($n = 16$, two independent experiments in duplicate for eight bacteria) were compared to untreated controls using independent students *t*-test. For the analysis of number of dents and ECS between treated bacteria and untreated controls a chi-square test was performed to evaluate number of bacteria with dents or ECS to number of bacteria without ECS. A p-value below 0.05 was considered significantly different. Data were plotted and analyzed in GraphPad Prism version 6.

3. Results

The efficacy of the inactivation treatments BPL, ethanol, formalin, NaOH, UV-C and pasteurization was examined, aiming to obtain intact inactivated bacteria. For this, eight anaerobic and facultative aerobic bacterial strains of different species from five phyla were selected based on their differences in cell wall structures, including two Gram-positive and six Gram-negative strains (Table 1).

3.1. Pasteurization is the least efficacious approach to inactivate all bacterial strains

To assess the efficacy of inactivation treatments, CFU/ml prior (at OD_{620} of 1.0) and post treatments were calculated by counting colonies from agar plates (Supplementary Table S1). The inactivation efficacy of BPL, ethanol, formalin, UV-C and NaOH was 100%, with no bacterial colonies observed on the plates. While 30 min of pasteurization inactivated >99.65% bacteria for all strains, Fig. 2A shows that some cells of *E. coli*, *S. typhimurium*, *P. distasonis*, *L. lactis* and *A. muciniphila* survived the pasteurization process, rendering this inactivation approach the least efficacious of all those tested. UV-C light treatment was very cumbersome due to evaporation of liquids in the flow-cabinet and therefore discarded as suitable method for standardized inactivation.

3.2. Maintenance of cellular integrity

We applied several complementary methods to evaluate the effects of the various inactivation treatments on cellular integrity of inactivated bacteria. We measured the release of extracellular DNA (*eDNA*) in the supernatants of all bacteria, labeled surface proteins with FITC, and labeled intracellular DNA with DAPI in four bacteria, and imaged a selection of two bacteria with SEM.

3.2.1. Inactivation with BPL and ethanol results in extracellular DNA (eDNA) similar to non-treated cells

Supernatants of untreated bacteria (served as negative control) generally contained low amounts of *eDNA* (mean ± standard deviation, 30.7 ± 17.5 ng/μl; Fig. 2B). Striking was the high *eDNA* (116.4 ± 33.3 ng/μl) released in all NaOH-treated samples when compared to the negative control ($p < 0.001$). *eDNA* in BPL-treated bacteria was consistently low. Here, the mean value (29.7 ± 11.3 ng/μl) was not statistically significantly different from the negative control (30.7 ± 17.5 ng/μl). When comparing pasteurization, ethanol and formalin with each other, the standard deviations of pasteurization (61.0 ± 18.9 ng/μl) and ethanol (42.5 ± 18.1 ng/μl) were smaller than that of formalin (57.9 ± 56.9 ng/μl). From all the treatments, the mean released *eDNA* concentrations of ethanol (42.5 ± 18.1 ng/μl) and BPL (29.7 ± 11.3 ng/μl) were closer to and not statistically significantly different from the negative control (30.7 ± 17.5 ng/μl), and all their mean concentrations were under 50 ng/μl. The results of quantified *eDNA* in supernatants indicate that BPL and ethanol inactivate bacteria while keeping the cells intact. Pasteurization ($p < 0.01$), NaOH ($p < 0.001$) and formalin (ns; only for *A. muciniphila, F. nucleatum* and *P. distasonis*) lead to leakage of DNA and hence suggests impaired cellular integrity.

3.2.2. Inactivation with formalin and BPL does not result in DNA release or protein loss on the cell membrane

FITC labeling and DAPI staining were performed to examine the preservation of bacterial membranes of four selected bacteria: *A. muciniphila*, *E. coli*, *L. lactis*, and *S. gallolyticus* (Fig. 3). These bacteria were selected based on their different cell wall compositions and growth requirements. Both *A. muciniphila* and *E. coli* are Gram-negative. *A. muciniphila* is anaerobic and *E. coli* is facultative anaerobic. Both *L. lactis* and *S. gallolyticus* are Gram-positive and facultative anaerobic. During the staining process, FITC binds to outer surface proteins reacting with primary amine bonds to form a covalent link (Mao and Mullins, 2010; Riggs et al., 1958), while DAPI binds to AT rich regions in the DNA (Eriksson et al., 1993). Positive non-inactivated controls were labeled with FITC and DAPI or FITC-only to demonstrate positive labeling with FITC, and intact bacterial membranes (DAPI only binds to DNA when cells are permeabilized) (Glavin et al., 2004). Negative non-inactivated controls were treated with DAPI only (no DAPI-signal under microscope). Since both positive and negative controls were not inactivated in

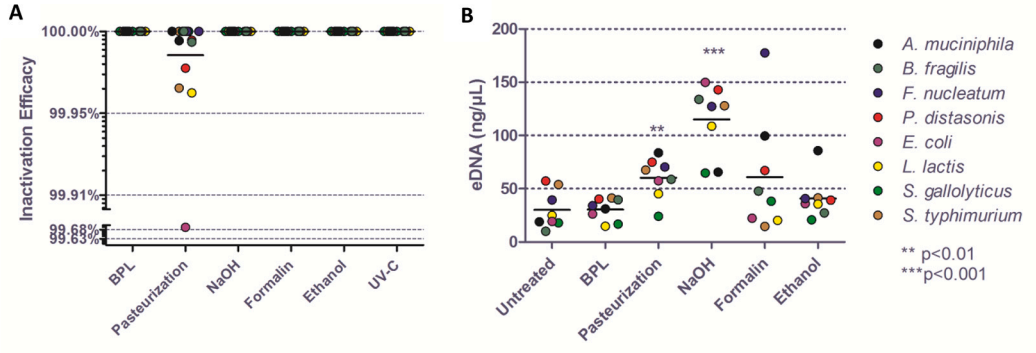

Fig. 2. A) Inactivation efficacy. Complete inactivation of bacteriai.e., 0 CFU/ml, was taken as 100%. Mean values calculated across all bacteria (displayed by horizontal blue line): Pasteurization 99.97 ± 0.07%, and 100% for untreated bacteria, BPL, NaOH, Formalin, UV-C and Ethanol. Pasteurization displayed colonies for *E. coli*, *B. fragilis*., *S. typhimurium*, *P. distasonis*, *L. lactis* and *A. muciniphila* compared to untreated control (n = 2 independent experiments in duplicate, the average value for each bacterium is plotted). B) Extracellular DNA (*eDNA*) release. Values were measured by Nanodrop, of which the mean was calculated across all bacteria (displayed by horizontal blue line): Untreated bacteria 30.67 ± 17.45 ng/µl, BPL 29.74 ± 11.34 ng/µl, Pasteurization 60.96 ± 18.85 ng/µl, NaOH 116.4 ± 33.33 ng/µl, Formalin 57.88 ± 56.94 ng/µl, Ethanol 42.50 ± 18.14 ng/µl. NaOH and pasteurization displayed significantly higher *eDNA* release than untreated control. Independent students *t*-test between treatments of all bacteria combined (n = 16 (2 for each bacterium), the average value is plotted). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

any way, the excessive movement of bacterial cells observed during microscopy did not allow us to yield sharp pictures (results not shown). The inactivated bacteria are shown in Fig. 3A (FITC) and 3B (DAPI).

We observed intracellular DAPI signal in bacteria treated with BPL, ethanol, formalin, and pasteurization which confirms that the DNA was retained in the nucleus. In contrast, limited DAPI-staining was observed with NaOH-treated cells, indicating that DNA escaped the nucleus and bacterial cells. FITC-labeling was not affected by any of the inactivation methods for *S. gallolyticus* and *A. muciniphila,* however, FITC labeling was ablated or reduced for *E.coli* and *L. lactis* inactivated with NaOH and *L. lactis* inactivated with ethanol and pasteurization.

To conclude, while BPL and formalin inactivation treatments preserved the cell membrane proteins in all bacteria (FITC staining) and did not result in reduced DAPI signal (DNA release), NaOH inflicted considerable damage to the membrane, releasing the DNA (DAPI staining) or even lysed bacterial cells completely (*E. coli*).

3.2.3. Imaging of bacterial surface by scanning electron microscopy (SEM) shows limited effect on cell integrity of BPL and 70% ethanol

To visualize whether surface structures of inactivated bacteria were intact or damaged, SEM imaging was carried out. We investigated BPL and ethanol treatments using SEM imaging because they had 100% inactivation efficacy and limited *eDNA*. Since NaOH had a relatively high *eDNA* in most bacterial supernatants (Fig. 2B) and reduced DAPI-staining, we included this treatment as a positive control of reduced cellular integrity. The Gram-positive facultative anaerobe *S. gallolyticus* and the Gram-negative anaerobe *A. muciniphila* were selected for visualization with SEM because their *eDNA* release was consistently below 100 ng/µl and were not affected by any of the methods in their FITC-labeling or DAPI-staining.

As shown in Fig. 4A and E, *A. muciniphila* are oval-shaped whereas *S. gallolyticus* forms diplococci. Normal untreated as well as treated bacteria show the division ring. As expected, the NaOH-treated *A. muciniphila* and *S. gallolyticus* bacteria displayed the most severe structural damage (27.6% dents in surface for Am, 64.9% for Sg) compared to the controls (9.1% for Am, 27.6% for Sg) (Table 2; Fig. 4D and H), even though they had the lowest *eDNA* release with NaOH. Ethanol-treated *A. muciniphila* exhibit increased extracellular surface structures (ECS) in 30.4% of cells (ECS, arrows in Fig. 4B) compared to 4.5% untreated cells (Fig. 4A), whereas there was no difference in ECS between non-treated and treated *S. gallolyticus* cells. No significant difference (Chi-square statistics) was observed in the number of dents between ethanol-treated and control cells for both *A. muciniphila* and *S. gallolyticus* (Fig. 4B, and F). BPL-treated *A. muciniphila* are preserved, while *S. gallolyticus* shows slightly more dents, however, only 8 cells could be quantified for this condition, and these structural dents resulted in limited *eDNA* release and no change in DAPI-signal. Hence, BPL and 70% ethanol are demonstrated to generate inactivated bacteria with minimal effect on cellular integrity of *S. gallolyticus* and *A. muciniphila* (Fig. 4B–C, F–G).

4. Discussion

We aimed to examine different methods for inactivation of bacteria while preserving bacterial cell integrity. We chose eight bacteria from five phyla with different cell wall compositions (Gram positive and Gram negative) and oxygen requirements to inactivate with BPL, pasteurization, ethanol, formalin, and NaOH. Inactivation was confirmed by CFU counting, and FITC/DAPI staining and *eDNA* quantification were carried out to examine the cellular integrity of bacteria. Additional experiments with SEM were performed to visualize and confirm cellular integrity of 2 selected bacterial strains. Our results demonstrate that BPL and ethanol showed 100% inactivation efficacy and minimal leakage of DNA, while BPL and formalin showed most consistent FITC-labeling and DAPI-staining. These methods may be applied in a standardized inactivation protocol to study for examples host-microbe interactions with long incubation times (Taddese et al., 2020). Pasteurization resulted in incomplete inactivation and was therefore not suitable for standardized inactivation considering multiple bacterial strains form different species. Although UV-C light very efficiently inactivated all bacteria, the procedure to generate inactive bacteria was impractical and was therefore not further investigated. UV-C light is however a method that is used in the (animal) food industry (Blazquez et al., 2017; McLeod et al., 2018), and might be suitable when investigating single bacterial species.

While formalin fixation retained FITC-labeling and DAPI-staining of *A. muciniphila*, *S. gallolyticus*, *L. lactis* and *E. coli*, considerable *eDNA* release was detected for *A. muciniphila* and *F. nucleatum*. This may indicate that formalin can disrupt the structures of certain bacteria more than others, potentially making this treatment unreliable for generalized application. Formalin is the most frequently used fixation agent for histological tissue samples. Formalin interacts with nucleic acids and proteins and dehydrates cells while preserving surface structures (McDonnell and Russell, 1999). It is therefore a very suitable method when applied for microscopy techniques. However, due to the cross-links created between proteins that could change epitopes for

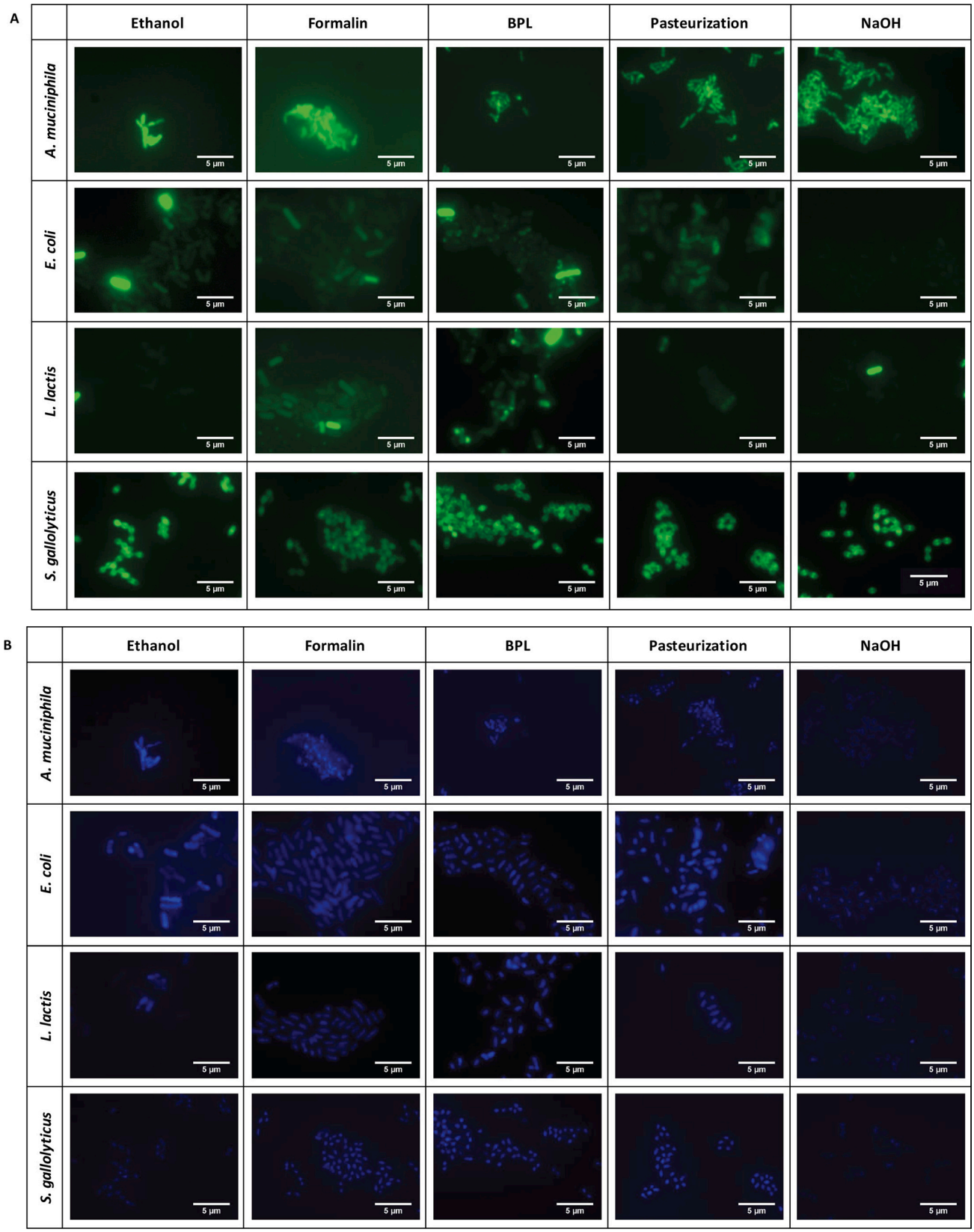

Fig. 3. FITC-labeling (A) and DAPI-staining (B) of inactivated *A. muciniphila*, *E. coli*, *L. lactis*, and *S. gallolyticus*. Inactivation treatments are stated on the columns, the rows represent the different bacteria. FITC-labeling is seen in green and DAPI-staining in blue. Non-inactivated controls are not included in the figure (see text), but only scored under the microscope. (n = 2 independent experiments, representative pictures are shown at 100× (oil immersion)). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

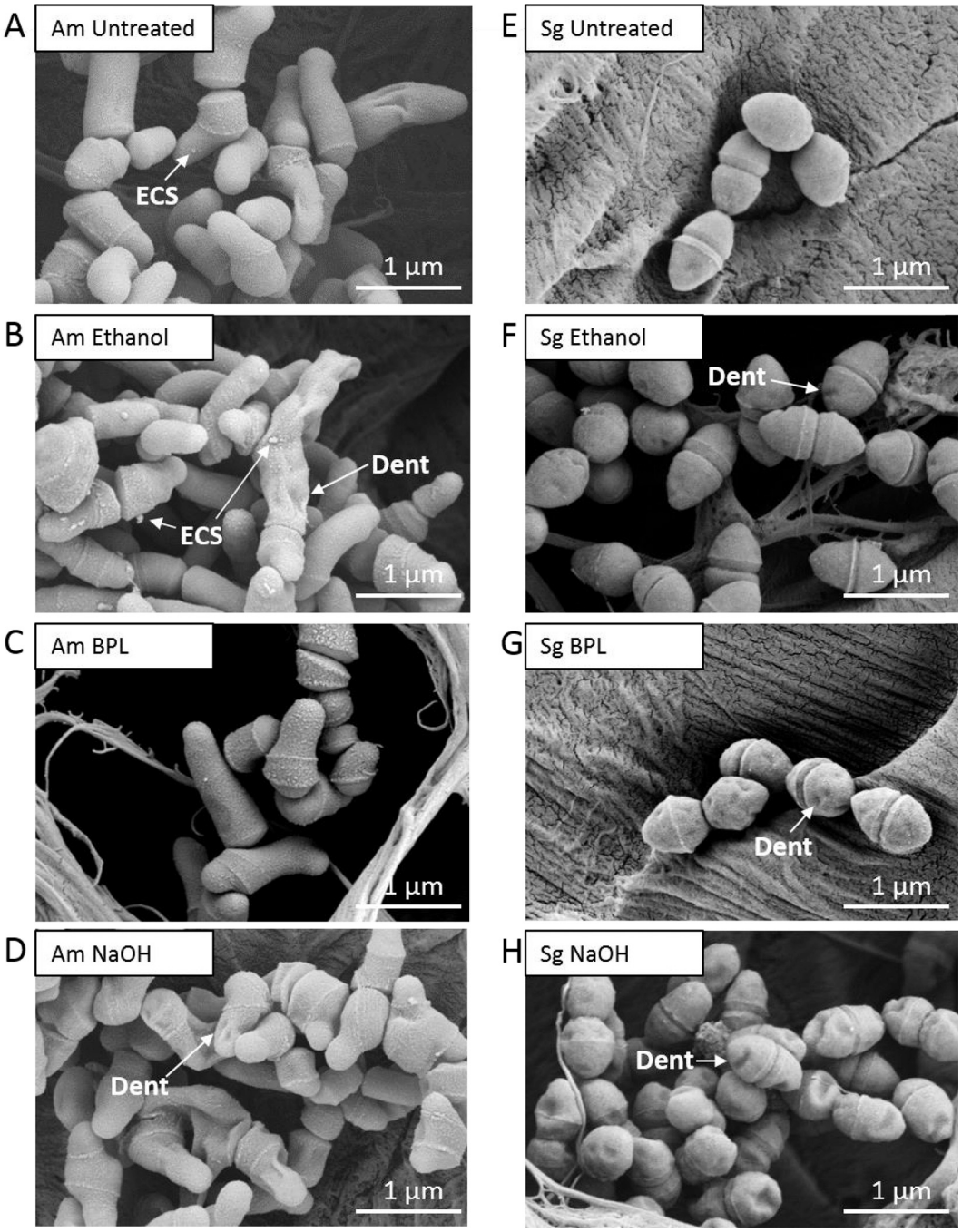

Fig. 4. SEM images of untreated and inactivated *A. muciniphila* (Am) and *S. gallolyticus* (Sg). Shown are untreated Am (A) and Sg (E), ethanol-treated Am (B) and Sg (F), BPL-treated Am (C) and Sg (G), and NaOH-treated Am (D) and Sg (H). Dent-arrows point at damages on treated bacteria. Extracellular structures (ECS)-arrows points at extracellular structures on Am.

antibodies or protein structure (Vani et al., 2006), formalin-inactivation can be disadvantageous when studying host-microbe interactions or generation of antibodies for *in vivo* usage.

NaOH has been previously used to produce bacterial ghosts. The minimum inhibitory concentration of NaOH was determined to produce bacterial ghosts from *Staphylococcus aureus* (Vinod et al., 2015). Using scanning electron microscopy (SEM), it was demonstrated that NaOH perforates the bacterial membrane where the bacterial DNA escapes and is degraded. Except for the pores, the membrane and cell wall of the bacteria were shown to remain intact. Previous studies have shown that using NaOH perforates the bacterial cell wall, allowing the DNA and the cytoplasmic contents to leak out of the cell (Vinod et al., 2015; Wu et al., 2017). Similar protocols have been employed to generate ghosts form *Salmonella enteritidis* (Vinod et al., 2014). The effect of NaOH treatment depends on temperature, the presence of other proteins, the pH and the concentration of NaOH, (Starliper et al., 2015; Wedel et al., 2019). Varying these conditions can result in effective production of bacterial ghosts, or complete lysis of bacterial cells. To avoid the damage on bacterial membrane during inactivation, the minimal inhibitory concentrations (MICs) of NaOH for individual bacteria have to be determined beforehand when using this method. In our experiments, NaOH treatment inactivated all bacteria and the protocol was simple to carry out, but it had highest *eDNA* release, bacterial DNA escaped from all bacteria and surface structures exhibited many dents, suggesting that

Table 2
Quantification of SEM pictures.

	Quantification of dents				Quantification of ECS			
	Cells with 0 dents	Cells with ≥1 dents	Total cells	% of cells	Cells with 0 ECS	Cells with ≥1 ECS	Total cells	% of cells
Am Untreated	20	2	22	9.1	21	1	22	4.5
Am Ethanol	20	3	23	13.0	16	7	23	30.4*
Am BPL	12	0	12	0	11	1	12	8.3
Am NaOH	21	8	29	27.6	28	1	29	3.4
Sg Untreated	6	0	6	0	6	0	6	0
Sg Ethanol	21	9	30	30	30	0	30	0
Sg BPL	4	4	8	50*	8	0	8	0
Sg NaOH	14	24	38	63.2**	36	1	37	2.7

Am = *Akkermansia muciniphila*, Sg = *Streptococcus gallolyticus*. Significant * < 0.05 or ** < 0.01difference from untreated control using Chi-square statistics.

the conditions for several bacteria were not optimal to create bacterial ghosts with intact surfaces. Therefore, we suggest that NaOH-based inactivation is a good technique for generating individual bacterial ghosts whose intracellular contents are released (Langemann et al., 2010; Wu et al., 2017). But in case surface proteins are the point of focus, it should be noted that the released intracellular contents may interfere by sticking to the bacterial surface.

Pasteurization is a commonly used method in the food industry to extend shelf-life of products without destroying essential nutrients. Common bacterial pasteurization works by raising the temperature up to 70 °C for a maximum of 30 min (Cefai et al., 1990), denaturing proteins and cell membranes of bacteria (Plovier et al., 2017). While pasteurization can be easily performed, not all bacteria were inactivated in our experiments in contrast to previous reports (Plovier et al., 2017). This difference might be the result of a different experimental setup e.g., we used a heat block instead of a water bath and a larger volume for pasteurization, possibly interfering with the conductivity. Perhaps the inactivation efficacy might be improved by increasing the temperature and/or the incubation time, but this was not further tested.

BPL inactivated all tested bacteria and without *eDNA* release or changes in DAPI/FITC signal suggesting intact cellular integrity. BPL is commonly used for the inactivation of viruses for vaccinations. It acts mainly by damaging the DNA (Perrin and Morgeaux, 1995). Previous research has found that BPL chemically modifies membrane fusion proteins and antigen proteins on the surface of Influenza viruses whereas another study showed that protein structures and folding are not greatly affected in rabies viruses (Bonnafous et al., 2014; She et al., 2013; Toinon et al., 2015). BPL has been previously applied on bacterial cells, but it is not known if BPL affects the bacterial surface proteins. So a direct comparison between live bacterial cells and BPL-inactivated cells in host-microbe interaction studies could help shed light on the effect of BPL on cell surface proteins. A major advantage of the BPL treatment is that the protocol does not include a washing step after inactivation, so there is minimal loss of bacterial material. Thus, BPL inactivation can be used when dealing with low-input samples. The disadvantage is that BPL must be handled very carefully when conducting experiments. Mice experiments showed that BPL led to formation of carcinoma upon skin exposure and nasal cancer upon inhalation (Colburn and Boutwell, 1968; Garte et al., 1985; Sellakumar et al., 1987). Thus, BPL should be used only in a fume hood. After bacterial inactivation, BPL-treated samples are placed at 37 °C for 2 h to inactivate BPL. Then the samples are safe to use for further applications.

Ethanol and formalin completely inactivated all tested microbes within 5 min. Both these treatments were readily executed and may be performed in standardized inactivation protocols. Ethanol is generally used for cleaning surfaces and sterilization due to its bactericidal effects (Morton, 1950). It inactivates bacteria by disrupting the cell membrane, dehydrating the bacterial cells and denaturing proteins (McDonnell and Russell, 1999). Interestingly, spores are not inactivated by ethanol (McDonnell and Russell, 1999; Otzen et al., 2007). The ethanol treatment did not show *eDNA* concentrations higher than untreated samples. Note that here the absence of *eDNA* is not the result of DNA precipitation by ethanol, since no resuspended DNA could be detected, even after centrifugation of any potential precipitate (Supplementary Fig. S1). Moreover, DAPI staining of ethanol treated cells showed that the DNA remained concentrated inside the cells (Fig. 3). A disadvantage of ethanol may be the washing out of lipoproteins from the cell-surface, so this approach might be less suitable for antibody production or immunization (Chao and Zhang, 2011; Kniggendorf et al., 2011). Note that *L. lactis* FITC-labeling was reduced in ethanol treated cells, so the effect of ethanol might depend on the bacterial strain used for investigation.

4.1. Conclusion

This study for the first time compares different methods for bacterial inactivation, on a range of microbiota members, with an outlook to their potential use for standardized inactivation. We show that BPL, ethanol and formalin treatments generated inactivated bacteria with limited effect on cellular integrity. Cellular integrity was based on release of DNA and corresponding DAPI staining. DNA release was not assessed for individual bacteria, as our experimental set-up (two experimental replicates in duplicate for each bacterium) did not allow for comparison at the species level. The release of RNA, proteins or low-molecular weight cellular contents was not assessed, and should be investigated further when important for down-stream applications. Release of potassium is for example recorded for ethanol inactivated bacteria (Yao et al., 2014). Importantly, the stability of inactivated bacteria at room temperature is so far unknown. Therefore, we recommend aliquoted long-term storage at −80 °C, to prevent freeze-thawing cycles. These inactivated bacteria can be used for the analysis of cell response to bacterial outer-membrane structures (Taddese et al., 2020), and used as potential carriers for drugs or antigens (Ganeshpurkar et al., 2014). Intact inactivated bacteria of clinically relevant species may potentially also be used for vaccination purposes (Pace et al., 1998). For each microbial application, one of these methods can be selected depending on the type of analyses that are planned, because each inactivation treatment has its advantages and limitations. In general, BPL or ethanol treatments exhibit promising potential for standardization resulting in the least membrane damage and *eDNA* release for the bacteria tested in this study.

Author contributions

RT, BED and AB contributed conception and design of the study. RT performed experiments and wrote the first draft of the manuscript. All authors supervised and discussed experiments, contributed to manuscript revision, read and approved the submitted version.

Data statement

All data are available upon request to the corresponding author.

Declaration of interests

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

RT was supported by Radboud Institute for Molecular Life Sciences (RIMLS 014-058). BED was supported by the Netherlands Organization for Scientific Research (NWO) Vidi grant 864.14.004 and European Research Council (ERC) Consolidator grant 865694: DiversiPHI. AB was supported by the Netherlands Organization for Scientific Research (NWO) Veni grant 016.166.089.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2021.106208.

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