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A fluorescence *in situ* staining method for investigating spores and vegetative cells of Clostridia by Confocal Laser Scanning Microscopy and Structured Illuminated Microscopy

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Abstract

Non-pathogenic spore-forming Clostridia are of increasing interest due to their application in biogas production and their capability to spoil different food products. The life cycle for *Clostridium* includes a spore stage that can assist in survival under environmentally stressful conditions, such as extremes of temperature or pH. Due to their size, spores can be investigated by a range of microscopic techniques, many of which involve sample pre-treatment. We have developed a quick, simple and non-destructive fluorescent staining procedure that allows a clear differentiation between spores and vegetative cells and effectively stains spores, allowing recovery and tracking in subsequent experiments. Hoechst 34580, Propidium iodide and wheat germ agglutinin WGA 488 were used in combination to stain four strains of Clostridia at different life cycle stages. Staining was conducted without drying the sample, preventing changes induced by dehydration and cells observed by confocal laser scanner microscopy or using a super-resolution microscope equipped with a 3D-structured illumination module. Dual staining with Hoechst/Propidium iodide

differentiated spores from vegetative cells, provided information on the viability of cells and was successfully applied to follow spore production induced by heating. Super-resolution microscopy of spores probed by Hoechst 34580 also allowed chromatin to be visualised. Direct staining of a cheese specimen using Nile Red and Fast Green allowed *in situ* observation of spores within the cheese and their position within the cheese matrix. The proposed staining method has broad applicability and can potentially be applied to follow *Clostridium* spore behaviour in a range of different environments.

Keywords: Clostridium sp., spore, staining,, Hoescht 34580, Propidium iodide, wheat germ agglutinin 488, Nile red, Fast Green, CLSM, 3D-SIM.

1. INTRODUCTION

The non-pathogenic spore-forming Clostridia, whose natural habitat is soil, is less described in the literature than equivalent pathogenic species. Biochemical and technological interest in those species has increased however, as they contribute to anaerobic fermentation in silages of crops and other biomass resulting in biogas production (Teixeira et al., 2016). Furthermore, non-pathogenic Clostridia can also spoil a variety of food products (Su and Ingham, 2000; McHugh et al., 2017), mainly through production of gas and butyric acid. Although they do not cause illnesses nor outbreaks, the food waste resulting from the activity of this species is of concern for the food industry. Spores can persist for months and may constitute an issue due to their resistance to commonly used antimicrobials and physical treatments (Setlow, 2016; Evelyn and Silva, 2018). Consequently, several studies have focused on spore structure and conditions triggering spore germination (Kohler et al., 2017). Microscopy techniques are among the best tools for these studies.

Due to their small size, *Clostridium* spores are often observed by conventional electron microscopy, either in scanning or transmission mode, that has a high resolution (D'Incecco et al., 2015, 2018; Bassi et al., 2009; El Jaam et al., 2017; Trunet et al., 2017). A disadvantage of this technique is that it requires specific skills, since sample preparation procedures for specimen fixation have to be used This approach, however, involves dead vegetative cells and spores thus no *in vivo* studies can be performed. Furthermore, electron microscopy allows a detailed observation of structures but qualitative information is not available other than when x-ray analysis is employed. An indirect reason for choosing this technique, rather than optical microscopy, is probably the lack of suitable and user-friendly staining protocols for *Clostridium* spores. Moreover, the resolution limit of light microscopy has only recently been improved by the availability of 3D-Structured Illumination Microscopy (3D-SIM). This technique uses the same standard fluorophores used in conventional fluorescent light microscopy (Schermelleh et al., 2010). Despite its limitations, the staining protocol proposed by Schaffer and Fulton (1933) for detecting bacterial endospores by light microscopy is also a useful reference protocol for this technique. According to this protocol, spores are directly stained by malachite green and safranin on the microscope slide and a subsequent flaming step is applied to dry the sample before observation. This uncontrolled dehydration, however, causes structural damage of the specimen, especially in case of bacterial cells and it does not permit the observation of hydrated cells or spores in a living condition. In fact, malachite green stain penetrates within the spore core only after the coating and cortex layers break. An additional drawback of this protocol is that no specific probes were proposed for the observation of specific spore or cell structures and the red colour observed is only the result of the counterstaining. More recent fluorescent staining methods for bacterial endospores (Schichnes et al., 2006) also have some limitations, since they do not allow live cells to be visualised or for cells and spores to be uniquely differentiated. Finally, phase contrast light microscopy does not require sample preparation but light contrast causes a bad visualization of vegetative cells in favor of dormant spores characterized by an high refractive index.

Nowadays, the use of fluorescent probes in confocal laser scanning microscopy (CLSM) or super-resolution microscopy - 3D-SIM is the most rapidly expanding approach in biological sciences, as it allows high spatial resolution and 3D images to be reconstructed with the advantage that fluorescent probes can be applied on both fixed or live cells. CLSM allows imaging of thick specimen by optical sectioning and elimination of out of focus fluorescence using filtering. In addition, super-resolution microscopy breaks the diffraction barrier of light allowing the dissection of the inner architechture of subcellular structures.

The goal of this study was to develop a robust and easy-to-apply fluorescent staining technique suitable for visualizing and differentiating live/dead vegetative cells and spores of Clostridia through both CLSM and super-resolution microscopy. Selected fluorescent probes that can be used simultaneously, including Hoechst 34580 which is highly sensitive to chromatin states, were considered to explore possible combinations that can provide greater levels of information on the chemical nature of the stained components. The feasibility of bacterial staining in different conditions, such as within culture media and *in situ* on a cheese slice, was also tested using four different *Clostridium spp*.. The proposed protocols represent useful tools for obtaining information on both the morphological features and physiological status of the spores and vegetative cells of *Clostridium spp*. in their natural environment.

2. MATERIAL AND METHODS

2.1 *Clostridium* strains, growth conditions and spore suspension purification

Four different *Clostridium* strains were used for this study: a *C. tyrobutyricum* IN15b strain from the Institute of Sciences of Food Production collection (ISPA-CNR, Milan, Italy), *C. butyricum* DSM 10702^T and *C. beijerinckii* DSM 791^T strains both provided by the Deutsche Sammlung Von Mikroorganismen und Zellkulturen, Braunschweig, (Germany), and a *C. sporogenes* ATCC 3584^T strain from the American Type Culture Collection (U.S.A) . The strains were routinely cultured in

Reinforced Clostridial Medium (RCM) broth (Scharlau Microbiology, Barcelona, Spain) and incubated at 37°C in an anaerobic jar equipped with a anaerobic reagent kit (Anaerocult A,VWR, Leuven, Belgium). Spore suspension was obtained by inoculating 1% of a 24-h culture of *C. tyrobutyricum* culture in RCM broth, as previously described by D'Incecco et al. (2015). The culture in the anaerobic jar was maintained at 37°C for 4 d and for a further 15 d at room temperature. Then, spores were harvested by centrifugation (8,000 g for 10 min at 4°C), washed three times with sterile water and finally stored in water at 4°C until use. The presence of spores was confirmed and spore number quantified using a Burker's counting chamber; the spore count ranged from 3.0 - 3.2 × 10^6 cfu/mL.

2.2 Experimental cheese intentionally contaminated with *C. tyrobutyricum* spores

An experimental cheese was made at laboratory scale with the addition of *C. tyrobutyricum* spores for testing the efficacy of the fluorescent staining protocol. The cheesemaking facilities at The Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne, (AU) were used for this experiment following existing protocols (Ong et al., 2011b) with a few modifications. Commercial full-fat pasteurised milk (four litres) from a local shop was heated to 32°C in a thermostatic bath and inoculated with 1% (v/v) of *C. tyrobutyricum* IN15b spore suspension. Glucono Delta Lactone (1 g/L) (Sigma-Aldrich Pty Ltd, Castle Hill, Australia) was used as an acidifying agent and rennet (0.06 mL/L) (Hannilase L, 690 IMCU/mL, Chr. Hansen, Bayswater, Australia) was added to the milk with gentle stirring. Once coagulation was achieved, the curd was cut into small pieces with a knife and gradually heated up to 38°C for 60 min. The curd was kept at 38°C until the pH dropped to 6.1-6.2, then whey was drained off. When the pH reached approximately 5.4, the curd was milled and salted with 2.5% w/w of salt before being pressed. The cheese was then stored at 15°C for 1 month.

2.3 Staining protocol for Confocal Laser Scanning Microscopy and Super-Resolution Fluorescence Microscopy of *Clostridium* in culture medium

C. tyrobutyricum, *C. butyricum*, *C. sporogenes* and *C. beijerinckii* cultures were submitted to double cell staining with Hoechst 34580 (HO) (Invitrogen, Mulgrave, Victoria, Australia) and Propidium iodide (PI) (Invitrogen). The HO was prepared from its stock solution (10 mg/mL) in MilliQ water purified to a resistivity of 18.2 mΩ (Millipore, Billerica, MA, US) and diluted to a final concentration of 12 µg/mL. The PI was prepared from its stock solution (1 mg/mL) in MilliQ water and diluted to a final concentration of 10 µg/mL. A 200 µL sample of culture broth was centrifuged (5 min at 10,000 *g*) and the pellet was washed once with sterile 0.2 M phosphate-buffered saline (PBS) pH 7.4 (Sigma–Aldrich). After removing the supernatant, 100 µL of PI and 100 µL of HO were added to the pellet and mixed. The suspension was then incubated at 37°C for 30 min in the dark, followed by centrifugation (10,000 *g*, 5 min). The supernatant was then removed and 200 µL of agar solution (0.25 g/ 50 mL) at ~40°C was added to the pellet. The mixture in the tube was quickly vortexed, 10 µL of this suspension was then placed onto a microscope slide and covered with a glass coverslip (0.17 mm thick) that was flush with the sample and secured with nail polish. Samples were observed using an inverted confocal laser scanning microscope (Leica SP8, Leica Microsystems, Heidelberg, Germany) powered by solid state 488 nm and 638 nm lasers with a 63x (numerical aperture 1.4) oil-immersion objective. Super Resolution DeltaVision OMX V4 Blaze microscope (GE Healthcare/Applied Precision, Uppsala, Sweden) equipped with 3D-Structured Illumination Module (3D-SIM) was used only on *C. tytobutyricum* spores to assess the advantage of this technique. An oil immersion 60x objective (numerical aperture 1.42) was used. The refractive index of the immersion oil is 1.518 and the sample was covered with a glass coverslip of 0.17 mm thick. Hoechst 34580 was excited at a wavelength of 405 nm and Propidium iodide at 488 nm. The emission filters were set at 392 - 440 nm for the former stain and at 535 - 617 nm for the latter. Images were processed using the SoftWorx imaging software (Applied Precision)

or ImageJ software (NIH). 3D-SIM Images are presented as projections of whole cell z-stacks taken at intervals of 0.2 μm to a total depth of 6 μm.

A specific experiment was carried out to evaluate the cell behavior under thermally stressful conditions. A fresh culture of *C. tyrobutyricum* IN15b was split in two aliquots. One was kept under anaerobic conditions at 37 °C, the optimum growth condition for this culture, while the second was incubated at 55 °C for two hours. Both samples were then observed by CLSM at the end of two hour period of incubation. This experiment was carried out in triplicate and the images presented are representative of 10 images.

In another set of experiments, lectin wheat germ agglutinin Alexa Fluor conjugate (WGA 488; Invitrogen, Mulgrave, Australia) was used at the final concentration of 10 μg/mL for detecting glycoconjugated molecules on the surface of bacteria, as this stain specifically binds to N-acetyl-D-glucosamine and N-acetyl neuraminic acid (or sialic acid) on the cell membrane. The same incubation conditions were applied as above. WGA was excited at a wavelength of 488 nm and the emission filters were set at 497 – 520 nm.

2.4 Staining protocol for Confocal Laser Scanning Microscopy of *Clostridium* spores in cheese

The lipid-specific stain Nile red (Sigma-Aldrich) was prepared from a stock solution of Nile red (1 mg/mL), containing 0.8 mL/mL dimethylsulfoxide (DMSO, Sigma Aldrich) and diluted in MilliQ water to a final concentration of 0.1 mg/mL just prior to staining. The protein-specific stain Fast green FCF (Sigma-Aldrich) was prepared from a stock solution (1 mg/mL in water) and diluted to a final concentration of 0.1 mg/mL. Samples for CLSM observation were prepared from the intentionally contaminated cheese. Thin slices (2 x 2 x 1 mm) were taken from the cheese interior and soaked for 10 min by adding in sequence: Nile red, Hoechst 34580 and Fast green working solutions before washing with MilliQ water (Ong et al. 2011a). The stained cheese slices were placed on a microscope slide (ProSciTech, Thuringowa, Queensland, Australia), mounted with glycerol-based anti-fading agent (AF2, Citifluor Ltd., Leicester, London, U.K.) and secured with a

glass coverslip (0.17 mm thick) (ProSciTech). Samples were observed using an inverted CLSM from Leica Microsystem (Heidelberg, Germany), as described above. The excitation/emission wavelengths were set at 488 nm/ 520–590 nm for Nile Red and at 638 nm/ 660–740 nm for Fast Green FCF.

3. RESULTS AND DISCUSSION

3.1 Staining of *Clostridium* in culture medium

CLSM was successfully used as a versatile detection tool to develop protocols for the fluorescent staining of endospores and vegetative cells of Clostridia. The combination of Hoechst 34580 and Propidium Iodide (HO/PI) stains is novel for Clostridia and enables specific staining of nucleic acids and allows a concurrent test of membrane integrity. The blue fluorescent HO is able to cross the membrane and on entering the cell binds nucleic acids. It can be considered an effective measure of cell viability or live cell staining and, in our samples, HO stained either live vegetative cells or spores (Fig. 1). In contrast, PI passes through disordered areas of dead cell membrane and binds to DNA double helix to emit red fluorescence. This occurs only in dead or compromised cells. Consequently, when stained with HO/PI, vegetative cells of *C. tyrobutyricum* displayed either blue or red fluorescence when alive or dead, respectively. This double staining also allows the endospore within the sporulated mother cell to be distinguished when both fluorescent emissions were observed simultaneously and this appears as a blue spore within a red mother cell (Fig. 1). The method proposed showed 100% spore staining efficiency, since spores were always stained by HO or PI. Viability tests have been previously proposed for either lactic acid bacteria by immunodetection of intracellular proteins with species-specific antibodies or solventogenic clostridia by flow cytometry (Hannon et al., 2006; Patakova et al., 2014) but to the best of our knowledge there has been no prior application of these coupled stains to *Clostridium* species.

The proposed protocol for HO/PI staining was tested for specificity and efficacy on three other non-pathogenic Clostridia of importance in food (Silvetti et al., 2018): *C. butyricum*, *C. sporogenes* and *C. beijerinkii* (Fig. 2, 3 and 4). The staining proved to work similarly with all tested species and interesting morphological diversity between these species were revealed using this technique. Spores were oval and surrounded by whole vegetative material in *C. tyrobutyricum* and *C. butyricum* strains while *C. sporogenes* (Fig. 3) showed disintegration of vegetative material and this happened, to a lesser extent, for C. beijerinckii. Rainey et al., (2015) also showed the rapid lysis of the vegetative material in *C. sporogenes* after sporulation. *C. tyrobutyricum* (Fig. 1) and *C. butyricum* (Fig. 2) spores were in sub-terminal position within the dead mother cells that had a rod shape. The spores of both *C. sporogenes* and *C. beijerinckii* appeared different and were swollen within the cell (Fig. 4). As a characteristic trait, the vegetative material of the mother cell of *C. beijerinckii* looked like a long tail, as previously observed by Sirisantimethakom et al., (2016) for *C. beijerinckii* TISTR after a 60-h batch fermentation.

The HO/PI staining protocol allowed us to follow the behaviour of vegetative cells of *C. tyrobutyricum* when exposed to heat stress. The vast majority of vegetative cells at the exponential phase of growth (Fig. 5a, 5c) died after heat treatment at 55°C for 2 h and only a few were able to survive by developing an endospore (Fig. 5b). In contrast, neither dead cells nor spores could be detected in the sample kept at 37°C, the optimum growth temperature for this species and all cells remained in the live state staining blue (Fig. 5d). Similar behaviour was observed for *C. tyrobutyricum* during the vat processing of hard cheese, where the curd cooking step involves heating conditions close to those tested in the simple experimental system used here (D'Incecco et al., 2018). In the prior study, electron microscopy was adopted in order to achieve higher resolution and to detect initial signs of either cell sporulation or spore germination. The present experiment demonstrates, however, that information on these physiological processes can be obtained in a rapid and equally reliable way using the current fluorescent staining technique. This could be of great

importance when screening tests or routine analyses are required. Moreover, it offers the potential for further future development of automated screening tools.

Spore populations are often heterogeneous, with some spores having a prolonged dormancy compared to others (Wang et al., 2015). Therefore, screening a high number of samples gives more reliable information. Since, upon germination, spores become more susceptible to commonly used inactivation processes, the process of germination-induction has been recently discussed as a possible strategy for decontamination procedures for spore-forming bacteria (Kohler et al., 2017). A fast method to determine the vegetative cell *vs* spore status that is also able to differentiate the viability of the target bacteria would provide a helpful tool for monitoring the efficacy of the germination-induction or other eradication procedure.

The HO/PI staining protocol here proposed represents an improvement compared to the commonly used protocol of Schaeffer and Fulton (1933) (supplementary file 1) or to the phase contrast light microscopy (supplementary file 2) method often used to detect endospores (Yang et al., 2009; Trunet et al., 2017). In fact, HO/PI staining can be performed directly on the microscope slide without killing the cells and avoiding the potential artefacts induced by drying. Furthermore, bacteria can be stained in the culture tube. The latter condition makes it possible to recover pre-stained bacteria allowing their use in 'doping' experiments. Cells and spores labelled by staining can potentially be tracked in different systems, such as food or feed. The recovery of stained bacteria is an important option that was not possible with the staining protocols of Schaeffer and Fulton (1933) or Schichnes et al., (2006) (supplementary file 3) , as the eating step results in adhesion of bacteria to the glass slide. Phase contrast light microscopy allows a good visualization of dormant spores only, while vegetative cells and spores under different physiological status are poorly visualized due to their high water content (Yang et al., 2009). This approach does not kill the spore but it is not compatible with samples other than pure culture. The method presented here is also an improvement with respect to the fluorescent method proposed by Schichnes et al., (2006), where the sample is dried on a heating block, treated with methanol, acetic acid, ethanol and finally

stained by Acridine Orange. Overall, the staining method proposed here gives live/dead information on the sample, allows *in vivo* and *in situ* studies and can benefit from the higher resolution of CLSM and super-resolution microscopy with respect to light microscopy, providing a clear advantage over previous techniques.

Some morphological features of *C. tyrobutyricum* were also probed by combining lectin WGA 488 with the double HO/PI staining procedure. Lectin WGA 488 binds to bacterial structures containing N-acetylglucosamine and N-acetyl neuraminic acid (or sialic acid) residues (Monteiro, et al., 2015), thus allowing the peptidoglycan layer to be highlighted. The detection of the three channels separately allows visualisation of (Fig. 6): the endospore (blue), the glycosylated layers (green) surrounding the endospore or mother cell and the dead mother cell (red). The layer surrounding the endospore (Fig. 6c) is known to contain multiple layers of the exosporium, coat and cortex that protect the spore. This layer was continuous and thicker compared to the layer surrounding the mother cell, consistent with the protective role of the spore coating (Setlow, 2016). The peptidoglycan layer has been seen to assume diverse architectures as a consequence of different growth/division processes of the cell (Wheeler et al., 2011). The advantage of the triple staining is that different cellular compartments and different physiological aspects of the spore and cell can be monitored.

The HO/PI/WGA488 triple stain provided further details of the cell and its intracellular compartments when the specimen was examined using a super-resolution structured illuminated microscope. The resulting 3D images show the condensed bacterial DNA within the endospore (Fig. 7) that instead looked like a larger blue diffuse spot by CLSM. Spore coat layers were well developed, whereas the cell mother wall was almost completely lysed, resulting in green-fluorescent fragments free in the medium (Fig. 7a). The highly specific nature of the fluorescence of HO dye allows the conformation and chromatin state within cell to be probed, potentially allowing more detailed future studies of the progression of sporulation under varied conditions. The potential of Hoechst dyes has been highlighted also for eukaryotic DNA (Wang et al., 2015). As expected, the

image quality was improved and more detailed information could be obtained when super-resolution structured illuminated microscopy (SR-SIM) was used, compared to CLSM. Whilst CLSM represents an easier approach when checking the presence of spores or monitoring changes in the colony, super-resolution microscopy allows more detailed higher resolution studies. Interestingly, this last technique could be applied to follow different stages of the sporulation process. The resolution for optical microscopy is limited by the diffraction of the light wave when focused on the sample. In contrast, SR-SIM illuminates the entire field with a striped pattern of light (Gustafsson, 2000), so improving the spatial resolution by a factor of two (Galbraith and Galbraith, 2011). When this excitation pattern mixes with the spatial pattern of the sample, an interference pattern is produced that is coarser than either pattern taken individually. This illumination pattern can be mathematically extrapolated to gain access to the higher resolution information within the sample. The 3D-SIM super-resolution imaging gave an improvement in resolution, thus allowing the DNA nanostructure within the spore to be observed.

3.2 Staining of *Clostridium* in cheese

The Hoechst 34580 dye (ie HO) was used together with Nile Red and Fast Green, to observe spores *in situ* within the matrix of a semi-hard laboratory scale cheese. Spores were added to the milk during cheese making and after 1 month of ripening at 15^{o}C triple staining was performed on thin cheese slices (Fig. 8). The *in situ* staining procedure was successful in revealing the protein and fat microstructure of the cheese together with the spores. This was expected as no alterations to the standard cheese staining protocol such as incubations or sample treatment procedures were included in the triple stain method. Spores were most often observed within the protein matrix, possibly due to their entrapment within the casein gel during coagulation. Several spores also appeared clustered together allowing the presence and spatial distribution of endospores to be examined.

This *in situ* staining of spores within a cheese matrix illustrates the potential use of this staining technique. We envisage that this could be extended, however, to other samples and matrices of

interest, be they soil or fermentation substrates in which Clostridia grow. The technique may therefore have broad applicability across several fields.

4. CONCLUSIONS

An easy-to-apply, rapid and robust protocol has been developed for multiple staining endospore-forming Clostridia. This staining also allows live cell imaging and when coupled with super-resolution microscopy, specific labelled DNA nanostructures can be clearly observed within endospores. We specifically focused on vegetative cells behaviour, induced by heat treatment, which can trigger cell sporulation response. The simplicity of the dual HO/PI staining and the clear differentiation between cell and spore obtained, make fluorescence microscopy and in particular super-resolution microscopy an useful tool for studying endospore-forming bacteria such as Clostridia. This method has the potential to allow the fast evaluation of Clostridia sporulation in a naturally occurring environment, such as a culture or *in situ* within a cheese. The technique may also lead to a better understanding of the behaviour of Clostridia in food processing and during storage allowing preventative and interventional strategies. The technique developed may also be applicable to the broader study of Clostridia in a range of other environments.

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Figures

Fig. 1. CLSM images of *C. tyrobutyricum* IN15b double stained with Hoechst 34580 (HO) and Propidium Iodide (PI). Individual stains are shown, together with the superimposed signal for double staining. Live cells were captured in fresh culture while dead cells and spores were captured at the death phase at the end of the growth curve. The scale bars are 3 µm in length.

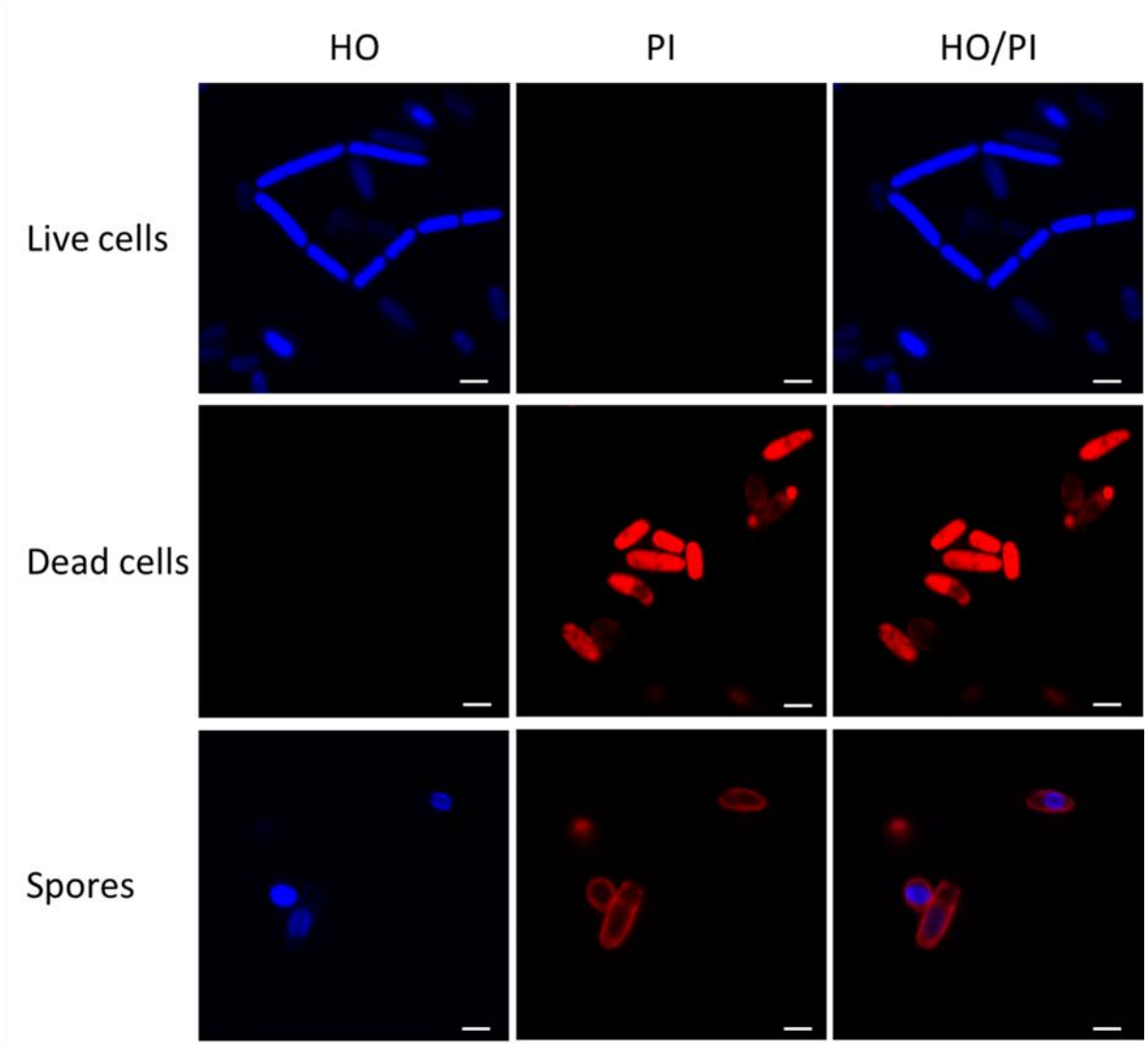


Fig. 2. CLSM images of *C. butyricum* CL30 double stained with Hoechst 34580 (HO) and Propidium Iodide (PI). Individual stains are shown, together with the superimposed signal for double staining. Live cells were captured in fresh culture while dead cells and spores were captured at the death phase at the end of the growth curve. The scale bars are 3 µm in length.

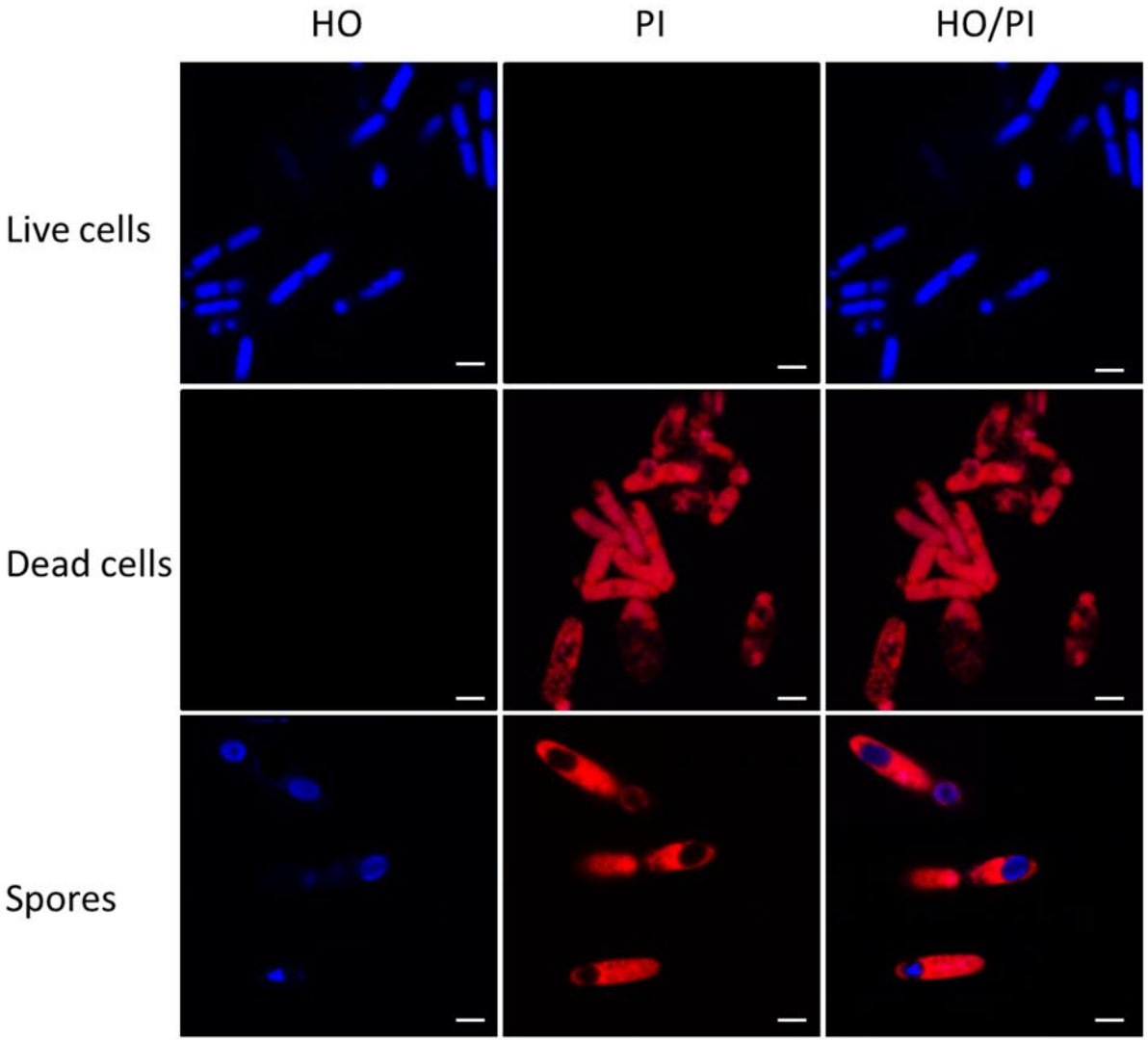


Fig. 3. CLSM images of *C. sporogenes* CL25 double stained with Hoechst 34580 (HO) and Propidium Iodide (PI). Individual stains are shown, together with the superimposed signal for double staining. Live cells were captured in fresh culture while dead cells and spores were captured at the death phase at the end of the growth curve. The scale bars are 3 µm in length.

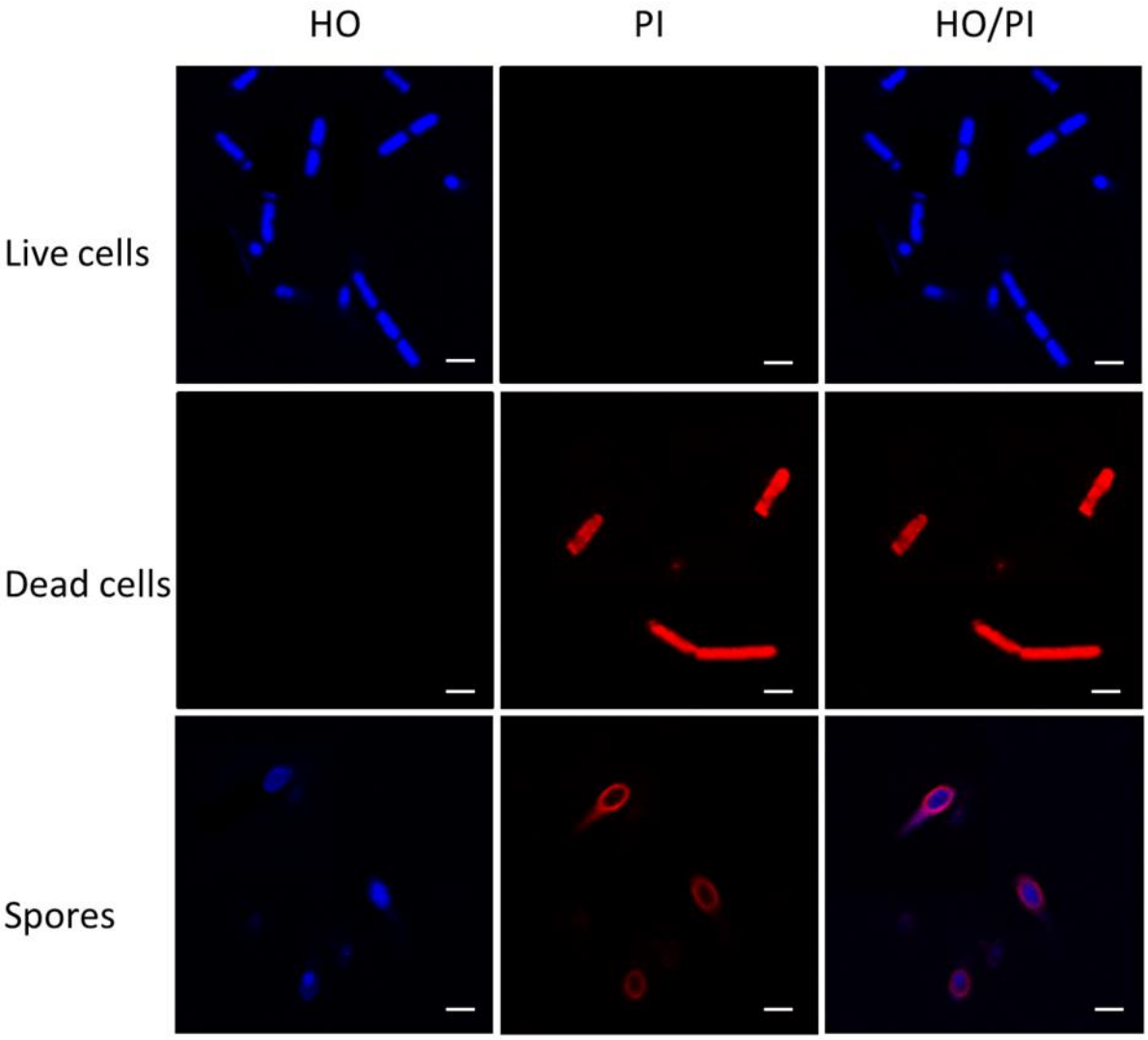


Fig. 4. CLSM images of *C. beijerinckii* CL28 double stained with Hoechst 34580 (HO) and Propidium Iodide (PI). Individual stains are shown, together with the superimposed signal for double staining. Live cells were captured in fresh culture while dead cells and spores were captured at the death phase at the end of the growth curve. The scale bars are 3 µm in length.

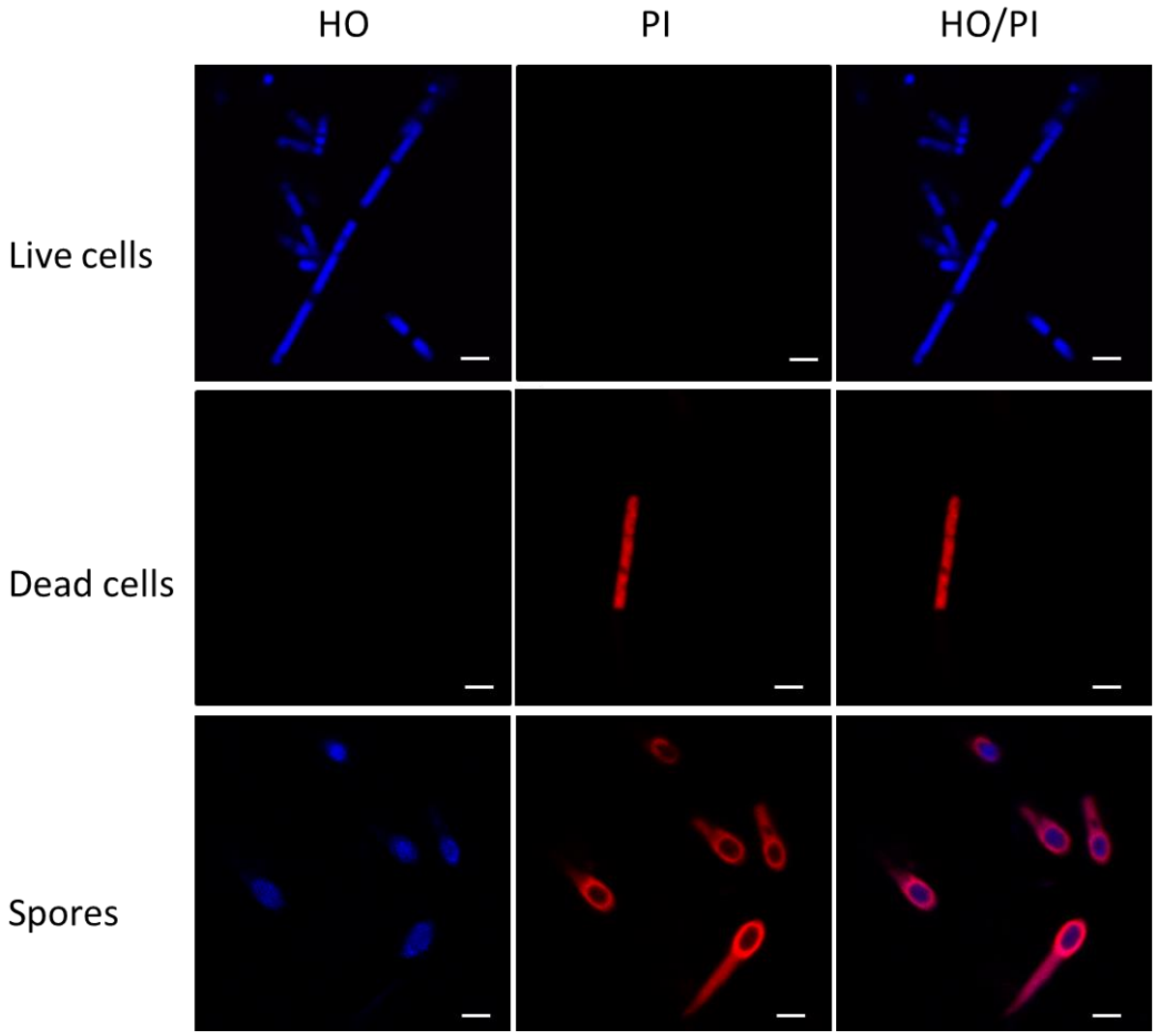


Fig. 5. CLSM images of HO/PI double-stained *C. tyrobutyricum* IN15b before and after heat treatment. Vegetative cells (panels A and C) were treated at 55 °C for 2 h (B) or vegetative cells were incubated at 37 °C for 2 h (D, control) before observation. Spores (S) were formed only after heat treatment.

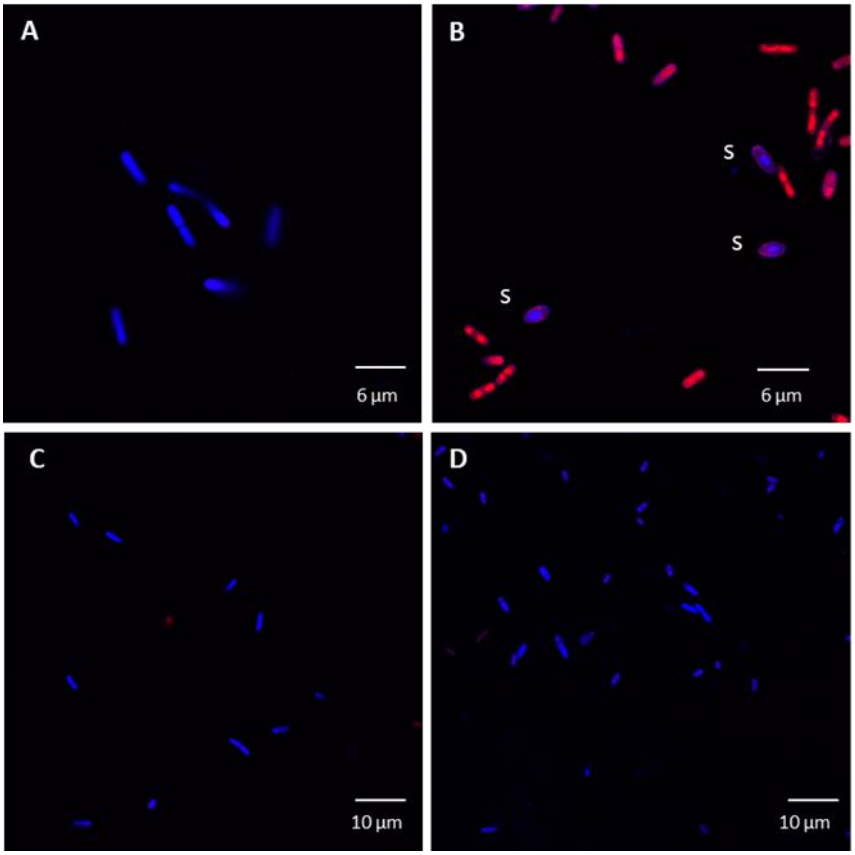


Fig. 6. CLSM images of *C. tyrobutyricum* IN15b triple stained with Hoechst 34580 (HO), Propidium iodide (PI) and Wheat germ agglutinin 488 (WGA). The images show individual PI staining of dead cells (a), HO stained live spores (b) and the WGA stained cell wall with N-acetylglucosamine and N-acetylneuraminic acid residues (c). Images from three channels (PI/HO/WGA) are assembled together in the merged image shown in (d). The scale bars are 3 µm in length.

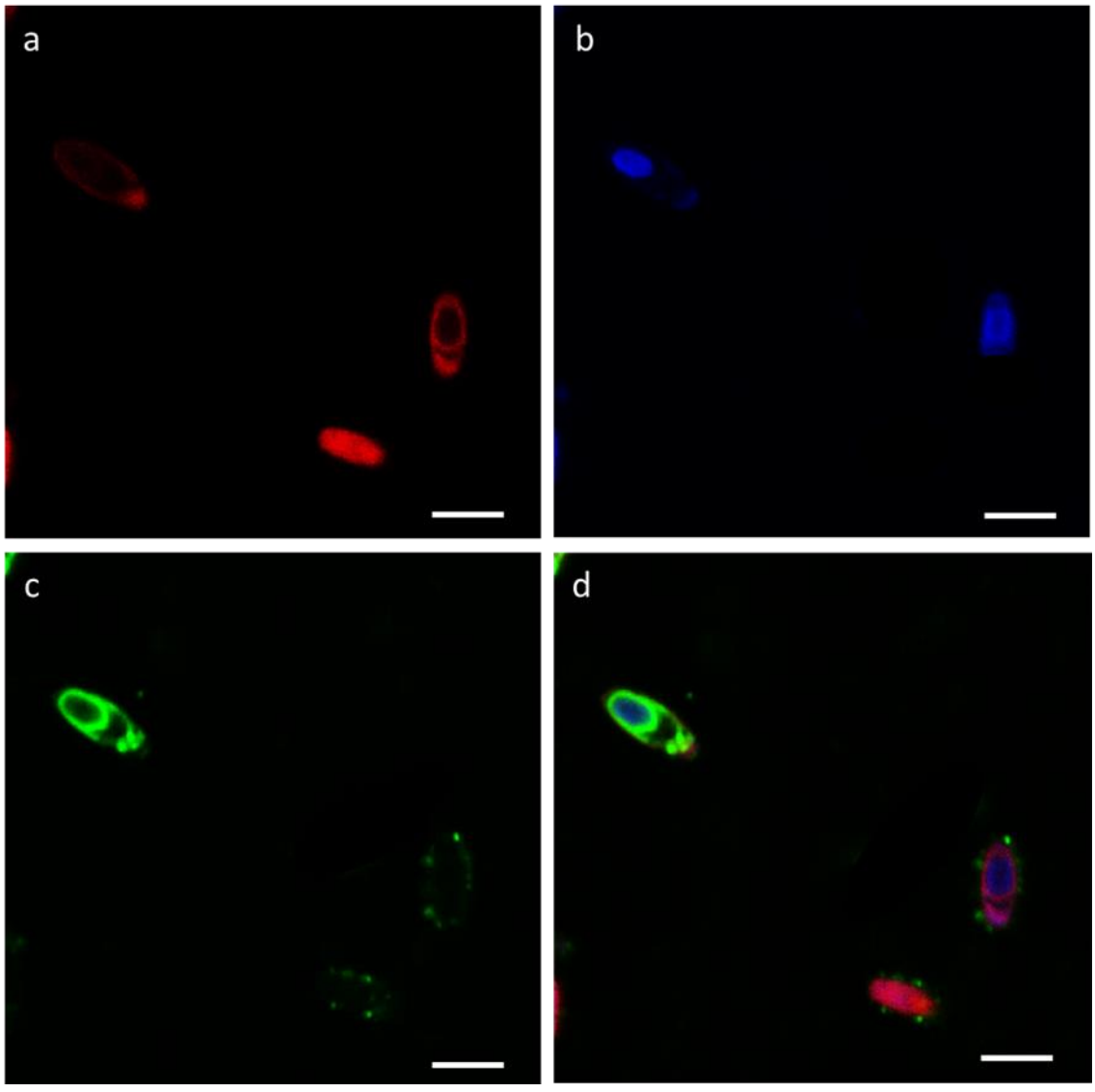


Fig. 7. Sporulated *C. tyrobutyricum* IN15b triple stained with HO/PI/WGA. 3D-SIM image (a) and optical section (b) showing a dead cell (red) containing its endospore whose protective layers (green) enclose the DNA (blue) within the space. The scale bar is 500 nm in length in panel a and 1 µm in length in panel b.

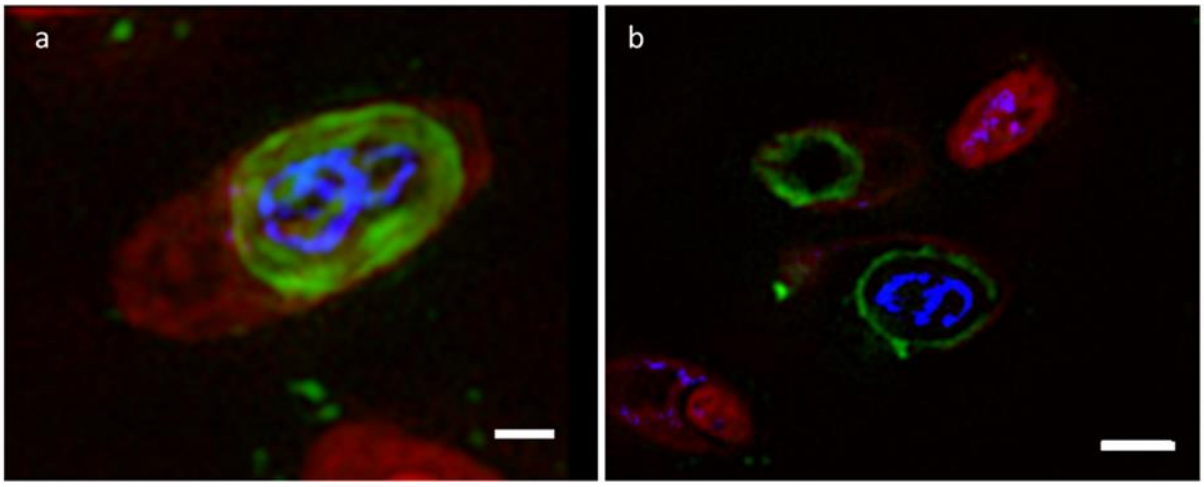
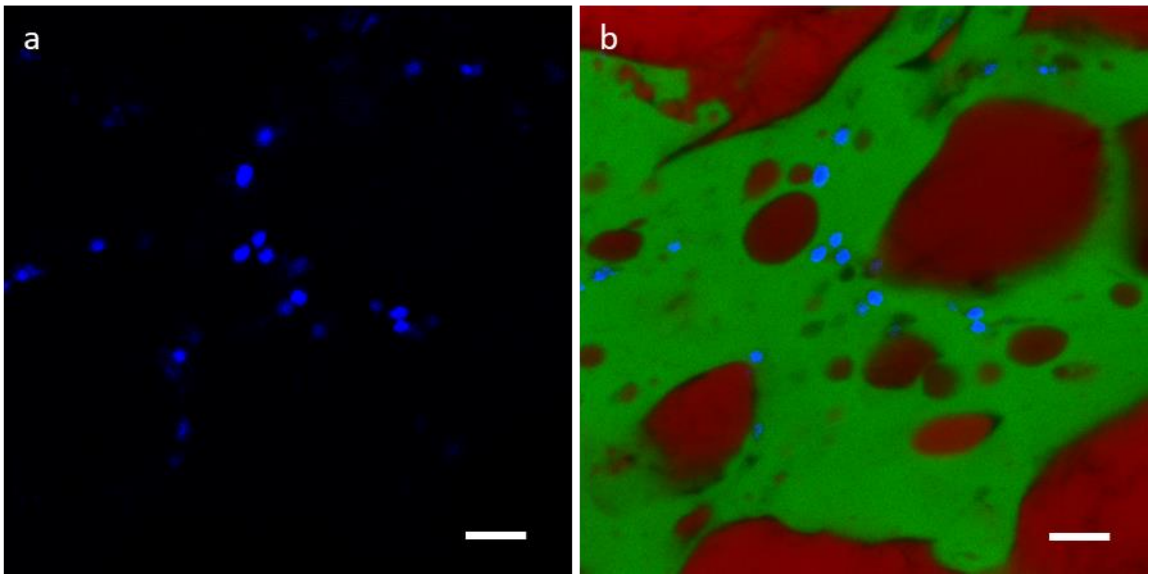
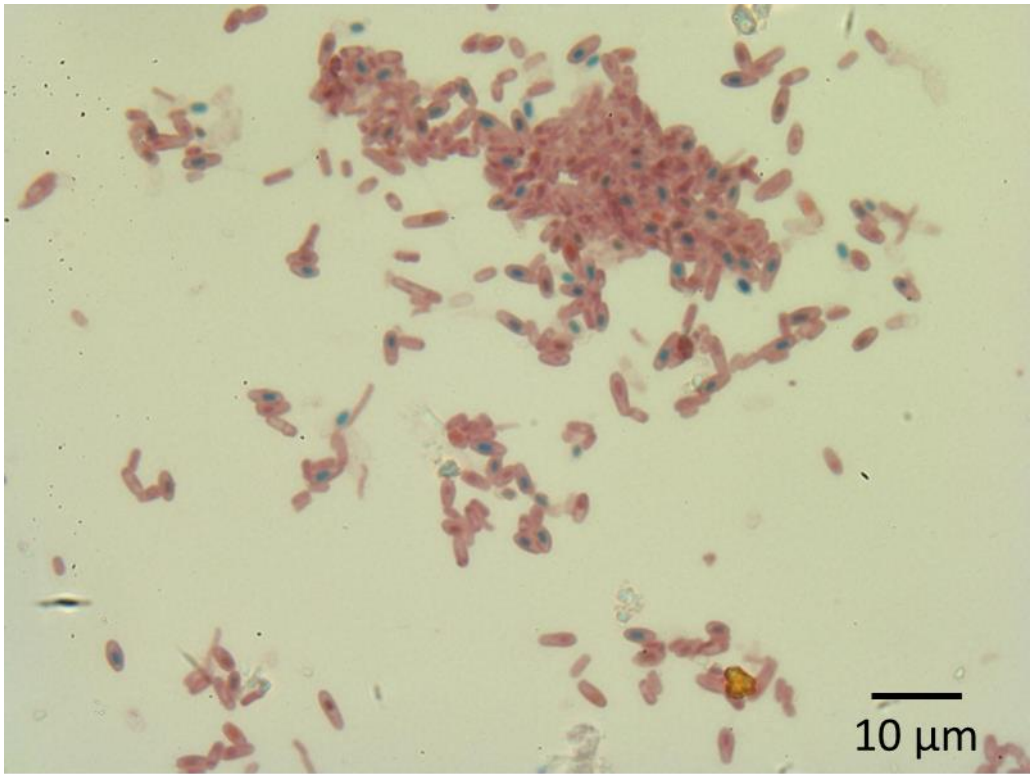


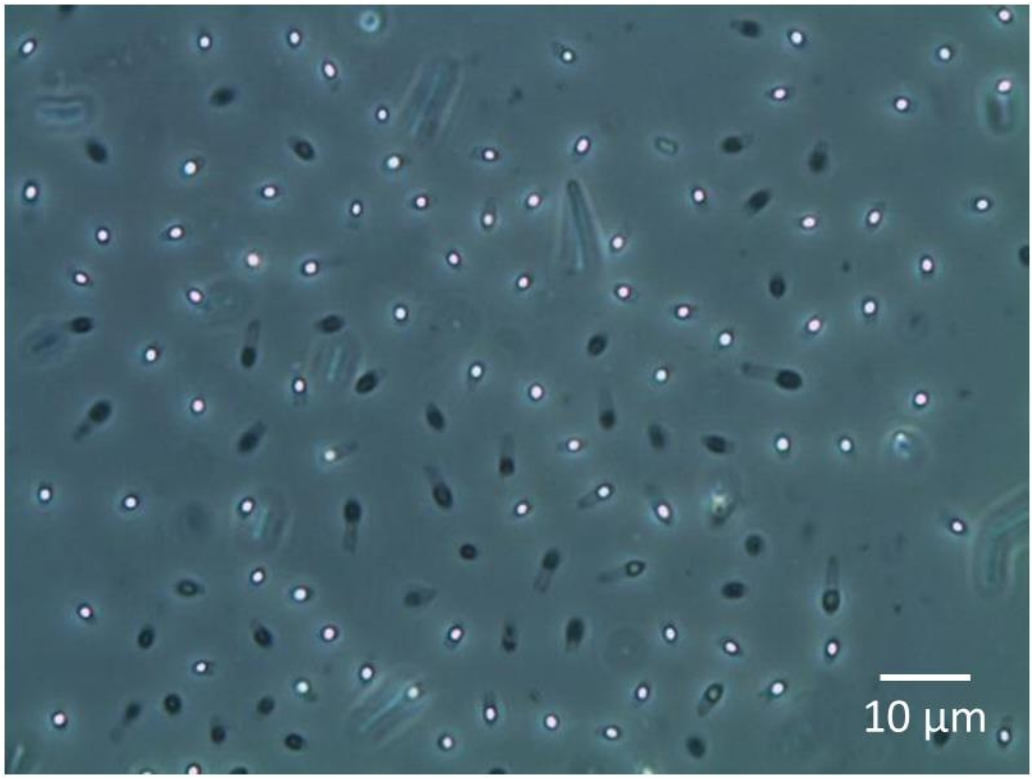
Fig. 8. CLSM image of spores in cheese triple stained with Hoechst 34580 / Nile red / Fast green. Spores appear blue, fat appears red and the protein appears green in these images. The scale bars are 3 µm in length.



Supplementary file 1. Light microscopy of *C. tyrobutyricum* stained by the method of Schaeffer and Fulton (1933). Spores are green due to malachite green staining and vegetative cells and some other spores are red because of the counterstaining by safranin. The difference between green- and red spores via this method is not clear. No live/dead information is available by this staining. At the time of staining, we killed cells and spores due to the high temperature reached. The high temperature allows malachite green to enter in the spore core. Cells are stuck to the slide; thus their recovery is not possible.



Supplementary file 2. Phase contrast light microscopy of spores without staining. Dormant spores look white because the core has a very low water content and thus a high refractive index. Dark spores have low refractive index probably because of germination. The advantage of this approach is that cells are not killed but live/dead status information is not available and vegetative cells are badly visualized. Also, this approach is not compatible with the detection of either vegetative cells and spores in a matrix (i.e. milk, cheese).



Supplementary file 3. Fluorescence microscopy of *C. tyrobutyricum* stained by applying the method of Schichnes et al., (2006). Acridine Orange stained both the cells and spores making their distinction not easy. With this approach, cells are destroyed by heating, methanol, acetic acid and ethanol. Cells are stuck to the microscope slide making their recovery not possible. Live/dead information is not provided by this technique.

