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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.

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

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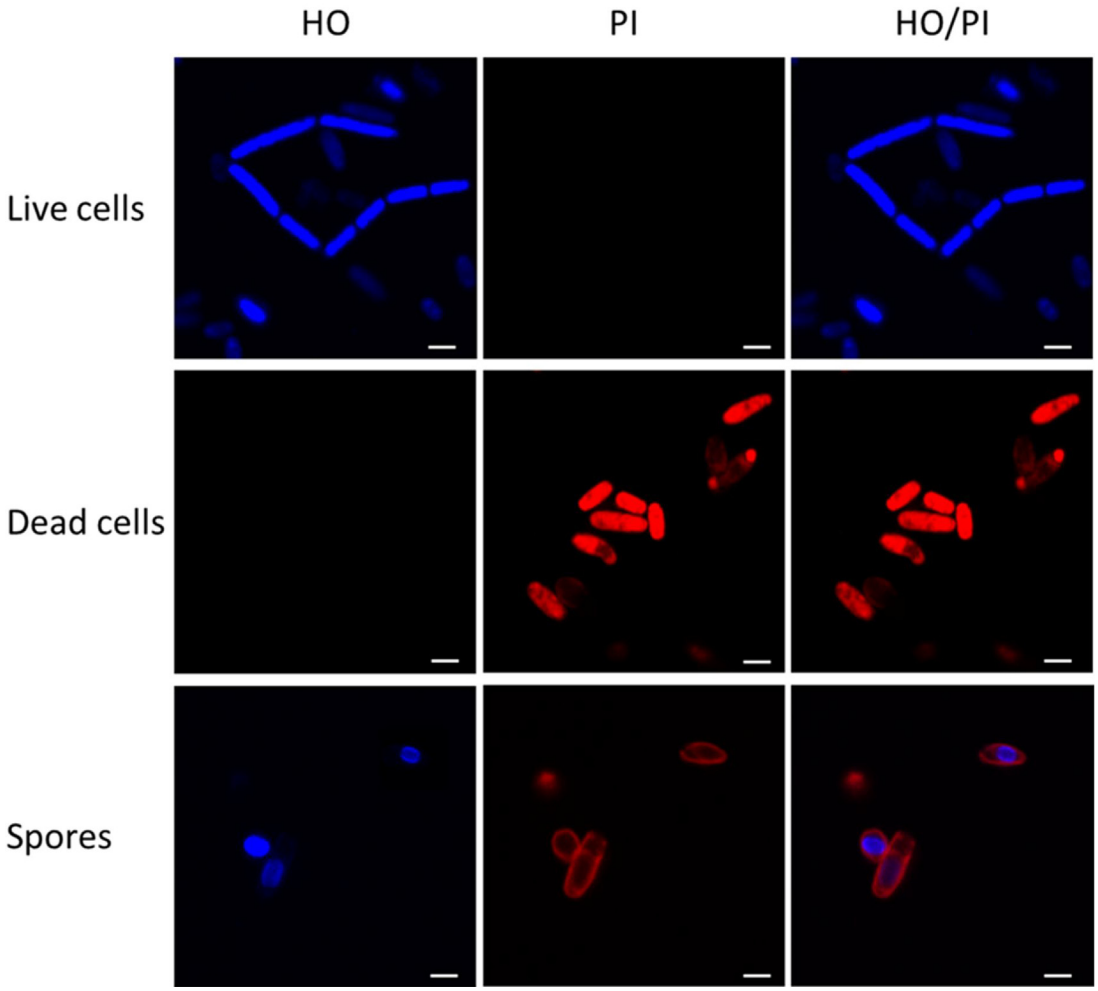

Fig. 1. CLSM images of *C. tyrobutyricum* IN15b double stained with Hoechst 34580 (HO) and Propidium Iodide (PI). Individual stains are shown, together with the superimposed signal for double staining. Live cells were captured in fresh culture while dead cells and spores were captured at the death phase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

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 Schaeffer 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.