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Review

Fluorescence-based tools for single-cell approaches in food microbiology

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ABSTRACT

The better understanding of the functioning of microbial communities is a challenging and crucial issue in the field of food microbiology, as it constitutes a prerequisite to the optimization of positive and technological microbial population functioning, as well as for the better control of pathogen contamination of food. Heterogeneity appears now as an intrinsic and multi-origin feature of microbial populations and is a major determinant of their beneficial or detrimental functional properties. The understanding of the molecular and cellular mechanisms behind the behavior of bacteria in microbial communities requires therefore observations at the single-cell level in order to overcome "averaging" effects inherent to traditional global approaches. Recent advances in the development of fluorescence-based approaches dedicated to single-cell analysis provide the opportunity to study microbial communities with an unprecedented level of resolution and to obtain detailed insights on the cell structure, metabolism activity, multicellular behavior and bacterial interactions in complex communities. These methods are now increasingly applied in the field of food microbiology in different areas ranging from research laboratories to industry. In this perspective, we reviewed the main fluorescence-based tools used for single-cell approaches and their concrete applications with specific focus on food microbiology.
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1. Heterogeneity as a driver of community functions

Advances in the field of the microbiology highlighted that contrary to the traditional view that pure-culture bacterial populations are clonal, isogenic bacteria can differ in a number of phenotypic characteristics (Brehm-Stecher and Johnson, 2004). Such variability dramatically affects the response of individual bacterial cells and the behavior of the whole population to environmental perturbations (Beaumont et al., 2009; Hamze et al., 2011; Lidstrom and Konopka, 2010; Ryall et al., 2012). For instance, it has been recently demonstrated that the phenotypic heterogeneity of *Salmonella enterica* cells contributes to the adaptive resistance of the population to several antibiotics through different molecule-dependent mechanisms (Sanchez-Romero and Casadesus, 2014).

The physiological adaptation of specific subpopulations to particular microenvironments constitutes one of the mechanisms underlying phenotypic heterogeneity. Indeed, food matrices mostly exhibit heterogeneous physicochemical composition and specialized phenotypes and strains can differentially occupy multiple niches demonstrating to be adapted to their local micro-environments (Gobbetti and Di Cagno, 2013). This is also noticeable when focusing on surface-adhered microbial communities known as biofilms, since the production of an extracellular matrix and the development of a three-dimensional (3D) architecture leads to the emergence of chemical gradients of oxygen, nutrients and metabolites (Stewart and Franklin, 2008). In such structured populations, bacteria can experience different growth conditions depending on their location in the 3D edifice and cells in the deeper layers, where oxygen and nutrient are depleted, can display modified activity in comparison to those living in the upper layers. As a consequence, these cells often display a higher resistance to antimicrobials due to different intertwined mechanisms including slow growth rate, induction of mediated stress response and modification of membrane composition (Bridier et al., 2011a; Hoiby et al., 2010).

Regardless of the environmental conditions, phenotypic variation can also arise from stochasticity in gene expression (Elowitz et al., 2002). Indeed, it has been shown that isogenic cells evolving under the same environmental conditions can show significant variation in molecular content and marked differences in phenotypic characteristics due to noise in gene expression (Kaern et al., 2005). Resulting cellular differentiation can provide the flexibility needed by the microbial community to be adapted to fluctuating environments or respond to abrupt stresses. Stochastic switching has been demonstrated in several phenotypic traits including persistence to antibiotics and lactose metabolism in *Escherichia coli* (Kuwahara and Soyer, 2012). Shimizu et al. (2011) quantitatively evaluated stochastic switching mediated adaptation by constructing an *E. coli* strain crucially dependent on the expression of

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a rewired gene essential for tryptophan biosynthesis. These authors experimentally confirmed the role of stochasticity in the adaptation of a starved bacterial population and the importance of the nutritional status and size of the population in this process.

Epigenetic events which generate inheritable phenotypic diversity without altering the underlying DNA sequence also participate in the formation of a heterogeneous population (Casadesus and Low, 2013). Overall, it can be considered as epigenetic, any transmittable information superimposed to the DNA sequence. Several converging and reinforcing signals, including simple feedback loops and complex DNA methylation patterns orchestrate this epigenetic inheritance (Casadesus and Low, 2006). For example, it has been reported that DNA methylation in subpopulations could impact bacterial resistance to antibiotics via the increasing of efflux pump expression and thus resulted in the production of heterogeneity in population with respect to antibiotics susceptibility (Motta et al., 2015).

Finally, population heterogeneity can also arise from a number of random or programmed genetic variations in specific subpopulations. Different mechanisms including spontaneous mutations, random transcription events, phage-related phenomena and mobile genetic elements such as plasmids and transposons can be at the origin of the emergence of variants in a population (Brehm-Stecher and Johnson, 2004). For instance, by focusing on the food pathogen *Listeria monocytogenes*, Van der Veen and Abee (2011) showed that the production of superoxide and hydroxyl radicals in continuous flow biofilms led to DNA damage and that the RecA-mediated mutagenic repair of the damaged DNA promotes the generation of genetic variants in the community. Moreover, the emergence of variants was correlated with an increase in rifampicin resistance in the bacterial community illustrating the community adaptation to the antibiotic stress.

To resume, the generation of heterogeneity appears as a ubiquitous and multi-origin phenomenon that constitutes a major adaptive or bet-hedging strategy of microbial populations (Beaumont et al., 2009; Lidstrom and Konopka, 2010; Ryall et al., 2012). The development of a realistic representation integrating this variability and complexity is thus absolutely required to better understand microbial community development and functioning. Moreover, some mechanisms responsible for key features of microbial populations can only be elucidated by investigating the taxonomy, physiology and/or activity of microbes at the cellular level. In this perspective, the development of single-cell approaches during the last years has driven the production of new data on the behavior of individual cells and thus enabled the development of new concepts in microbiology. In particular, the application of such new approaches helped to resolve fundamental questions in the field of food microbiology since they enabled, inter alia, detection, tracking and manipulation of bacteria at single-cell scale and thus led to a greater understanding of the in situ behavior of pathogens, their dynamic interactions in foods and the associated risks (Brehm-Stecher, 2013).

2. Fluorescent tools for single-cells analysis

Among methods used to investigate single-cell microbial phenomena, fluorescence-based approaches are of primary importance and encompass a wide variety of relevant techniques with many applications (Brehm-Stecher and Johnson, 2004). Fluorescence is a physical process associated with the emission of photons upon molecular transition from the electronic excited state to the ground state. The emission of photons occurs at a higher wavelength than the wavelength of the incident excitation source (Fig. 1A). Indeed, fluorescence is a fundamental tool compatible with the dynamic observation of living cells and the monitoring of metabolic activity, cellular components or gene expression in individual cells (Bridier et al., 2011b; Yao and Carballido-Lopez, 2014; Young et al., 2012).

2.1. Fluorescent markers

The increasing commercial availability of a wide variety of highly selective, sensitive and photostable fluorescent markers offers new possibilities in single-cell approaches. Table 1 summarizes some of the most commonly used fluorescent marker groups and their main applications. This includes fluorescent markers which target specifically some constituents such as carbohydrates, proteins or nucleic acid (Qin et al., 2007; Zippel and Neu, 2011), particular cellular states (Tawakoli et al., 2013; Webb et al., 2003), specific bacteria species (Rohde et al., 2015) and also fluorescent proteins (FPs) which are compatible with population or gene expression monitoring (Fleurot et al., 2014; Ma et al., 2011). In addition, the combination of different markers and/or FP-expressing strains coupled to multi-spectral excitation/detection of fluorescence enables simultaneous visualization of multiple cellular components, species and gene expression patterns at the single-cell scale (Courtney et al., 2012; Kitaguchi et al., 2005, 2006; Young et al., 2012).

Overall, we can virtually distinguish two classes of fluorescent staining. The first class includes markers that are lethal for the cells and consequently do not enable dynamic live observations of a given individual cell. Due to their low intrinsic fluorescence and high quantum yields when bound to nucleic acids, nucleic acid-binding markers were extensively used to stain bacteria in number of complex matrices including drinking water, dairy products and cheese or biofilms enabling successful subsequent cytometric detection or microscopic observation (Auty et al., 2001; Bridier et al., 2010; Bunthof et al., 2001a; Giao et al., 2009; Kahlisch et al., 2012). The *Bac*Light Live/Dead Bacterial Viability kit (Molecular Probes), composed of two fluorophores SYTO9 and propidium iodide (PI), constitutes for instance one of the most used fluorescent dying for cell viability assay and is based on the monitoring of membrane integrity. Such staining has been widely used to analyze the viability of bacteria in food matrix as reconstituted skim milk for example

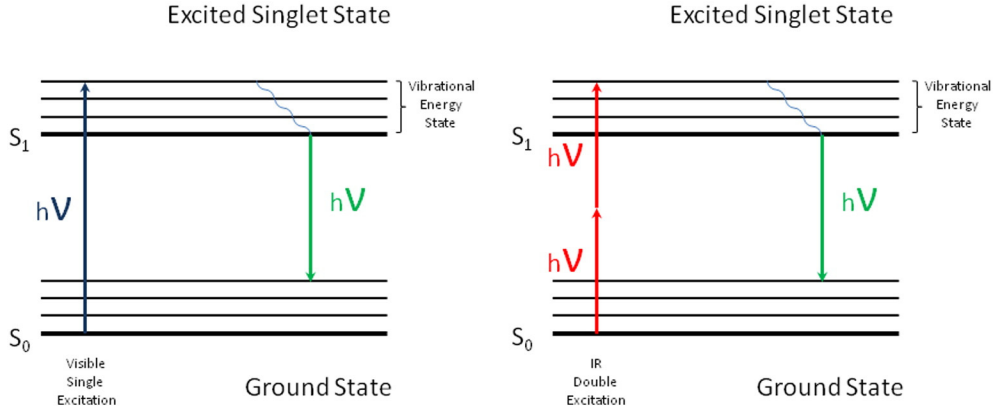

Fig. 1. Basic principle of fluorescence. Excitation modes: monophotonic excitation for WFM and C-LSM (linear phenomena), multiphotonic excitation for 2P-LSM (non-linear phenomena).

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