

# A rapid method for the assessment of the vitality of microorganisms using flow cytometry

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## ABSTRACT

Cultivability, viability and vitality make it possible to characterize the behavior of a cellular population. Vitality was assessed using the kinetic parameters of specific metabolisms depending on whether the strains were used, for example, for the acidification of lactic acid bacteria or for CO<sub>2</sub> production in fermenting yeasts. However, these methods are time-consuming. We developed a cytometric descriptor based on the energy-dependent extrusion of carboxyfluorescein from cells, subsequent to carboxyfluorescein diacetate staining, and compared it to the measurements of metabolic activities of various bacteria and yeasts. For all of the microorganisms tested, the cytometric descriptor  $\Delta FI_{15}$  was well correlated with the results of the metabolic measurements and, moreover, has the

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advantage of being easier and faster to use than metabolic methods. It can be very useful for evaluating the vitality of the starters before inoculation in industrial processes.

## 1 - INTRODUCTION

Some microorganisms are used in fermentation processes to produce various biomolecules such as organic acids, aldehydes and alcohols (white biotechnology). Lactic acid bacteria (LAB) and yeasts are widely used in fermented food products (e.g., dairy products, beverages, meats, vegetables). Regardless of the process, the viability and the activity (also referred to as vitality or dynamics) of the microbial starters must be high in order to quickly colonize the raw materials and avoid the growth of indigenous contaminants. Poor vitality may lead to delays in industrial processes and defects in products.

Cultivability, viability and vitality are three different descriptors that make it possible to characterize the behavior of microorganisms. Cultivability, the best-known property used by microbiologists, is defined as the ability of the cells to reproduce and form colonies on plate agar medium. Viability is defined by membrane integrity, protein synthesis and the energy production necessary to maintain cells (1). Hence, among a viable cell population, there are cultivable as well as non-cultivable cells that remain alive with intact membranes and potential metabolic activity (2, 3). Vitality is defined by the energy yielding capabilities of cells (4), by the dynamic behavior of a culture, or by the kinetic parameters of cells including the specific growth rate, the specific metabolite production rate or the specific substrate uptake rate.

In a microbial population, cultivable cells as well as dead cells and viable but non-cultivable cells may be present at the same time. Moreover, due to the heterogeneity of microbial populations, several cellular states with different metabolic activities can be distinguished among viable and cultivable

cells. Several authors revealed this heterogeneity using fluorescent probes and flow cytometry (FCM) or epifluorescent microscopy: for lactic acid bacteria in cider (5), in fermented meat (6), in whey (7), and in cheese (8). It is generally recognized that traditional reference culture-based methods (e.g., plate counts) do not guarantee the physiological state or the dynamics of the microbial population in terms of kinetics and metabolic activities. For example, yeast samples may reveal a low metabolic activity in a product, whereas its cultivability in the laboratory in terms of plate counts was good (9).

The measurement of the viability of microbial cells by FCM is usually based on the dual staining with propidium iodide (PI) (membrane integrity) and with fluorescein diacetate (FDA) or carboxyfluorescein diacetate (cFDA) (intracellular esterase activity). The fluorescent cells stained with FDA or cFDA is reported to correspond to the viable cells that have a metabolic activity and an intact membrane. Some cells lose their ability to reproduce but can still have an intact membrane and be stained by cFDA (2). Dual staining allows the differentiation of three sub-populations on the cytogram: dead cells (PI positive, cFDA negative = red cells), viable cells (cFDA positive, PI negative = green cells) and altered cells (PI positive, cFDA positive = green and red cells) (10, 11). Viable but non-cultivable cells are among this population of altered cells even if some altered cells may still be cultivable, depending on the ratio of green/red fluorescence intensities (12). The number of viable cells was simply assessed by FCM, which allows cell-by-cell analysis and gives a result in a very short time. However, the final fluorescence intensity of the viable cells depends on membrane integrity, intracellular esterase activity, intracellular pH and loss of fluorescent dye before analysis. Hence, fluorescence intensity alone cannot reflect vitality (13).

In order to assess the vitality i.e., the kinetic behavior of a microbial population, several methods based on the measurement of a specific activity can be used according to the metabolism of the microorganisms. They involve the determination of the lag phase time with respect to growth, acidifying activity, metabolite production rate and consumption rate. In two microbial populations with the same cellular dry weight or the same number of viable cells (specific activity), the time required to

reach a given optical density (OD), a given pH or a given quantity of product may be different according to the vitality of the two microbial populations. These methods provide some results about the whole culture and the overall cell population, but do not consider the heterogeneity within the microbial population. Moreover, they are time-consuming since it is necessary to grow a culture.

The three descriptors - cultivability, viability and vitality - are complementary: in order to ensure a reliable fermentative process, microorganisms must be of high quality, meaning that they must not only produce viable and cultivable cells in Petri dishes, but that they must also present a physiological state that guarantees good vitality. Starter quality has to be quickly assessed just before it is used in industrial processes.

Whereas viability can be quickly assessed by FCM, there is no equivalent method for vitality assessment. The aim of this study is to develop a flow cytometric descriptor that will allow to quickly assess the vitality of microbial cells, regardless of the strains. It is based on the observation that intracellular carboxyfluorescein (cF) is extruded after cFDA staining through an energy-dependent transport system. This phenomenon has been studied by several authors in bacteria and in yeasts: for *Lactococcus lactis*, the transport system is probably driven by ATP or a related compound, and strictly correlated with ATP concentration (14), and for *Saccharomyces cerevisiae*, the transport system is driven by membrane potential or proton gradient, depending on the activation of H<sup>+</sup>-ATPase (13). Regardless of the transport system, we hypothesized that the energy status of the stained cells can be assessed by measuring the efflux in standardized conditions. We then proposed to standardize a descriptor to stain cells and to quantify the carboxyfluorescein excreted from the stained cells, and to compare the results to the specific activities measured by different methods according to the types of microorganisms: acidifying rate for LAB (*Lactococcus lactis*), CO<sub>2</sub> production rate for fermentative yeasts (*Saccharomyces cerevisiae*), O<sub>2</sub> uptake rate for aerobic yeasts (*Yarrowia lipolytica*), and growth lag phase or energy charge for other microorganisms (*Staphylococcus aureus*, *Oenococcus oeni*).

## 2 - MATERIALS AND METHODS

### 2.1. Microbial strains and cultures

Two strains of *Saccharomyces cerevisiae* were used: the wine yeast L2056 (Lallemand SA, Blagnac, France), and the industrial ethanol yeast named Ethanol Red® (Lesaffre, Marcq en Baroeul, France). Yeasts were stored at 4°C on Sabouraud agar (Biokar, Allonne, France). Pre-cultures were performed in Sabouraud broth (Biokar, Allonne, France) for 24 h at 30°C, and cultures were inoculated at  $10^6$  or  $10^7$  cells.mL<sup>-1</sup>, depending on the assays, in an AF medium containing 180 g.L<sup>-1</sup> (or 200 g.L<sup>-1</sup>) of glucose (Univar, Fontenay sous Bois, France), 2.5 g.L<sup>-1</sup> of yeast extract, 0.3 g.L<sup>-1</sup> of K<sub>2</sub>HPO<sub>4</sub>, 0.5 g.L<sup>-1</sup> of KH<sub>2</sub>PO<sub>4</sub>, 2.5 g.L<sup>-1</sup> of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, and 0.5 g.L<sup>-1</sup> of MgSO<sub>4</sub> (all components from Thermofisher, Strasbourg, France) adjusted to pH 4.0. The temperature was set at 25°C for L2056, and at 30°C for the measurements in alcoholic fermentations with Ethanol Red®.

*Yarrowia lipolytica* CBS 6124 is a strictly aerobic yeast used in cheese. Pre-culture and culture were performed in 50 mL of Sabouraud broth (Biokar, Allonne, France) in a 250-mL flask, inoculated with  $10^6$  cells.mL<sup>-1</sup>, and placed on an orbital incubator at 100 rpm and 30°C for 24 h.

*Lactococcus lactis* ssp. *cremoris* SK11 was obtained from the culture collection of the Micalis Institute (<https://www.micalis.fr/>). Cells were grown at 30°C in 10 mL of M17-lactose (5 g.L<sup>-1</sup>) (Biokar, Allonne, France) for 7 h for pre-culture, before transferring 1 mL to a 100-mL flask of M17-lactose (5 g.L<sup>-1</sup>) for the culture. For the measurement of acidifying activity, the medium was 100 g.L<sup>-1</sup> dry skim milk (EPI-Ingredient, Ancenis, France), heat-treated at 110°C for 15 min in 150-mL flasks. The flasks were inoculated to reach a cell concentration of  $10^6$  cells.mL<sup>-1</sup>.

*Oenococcus oeni* MC1 was isolated from wine by Moët & Chandon. The pre-culture was performed in 10 mL of synthetic medium FT 80 broth (glucose: 5 g.L<sup>-1</sup>; fructose: 5 g.L<sup>-1</sup>; L-malic acid: 5 g.L<sup>-1</sup>; yeast extract: 5 g.L<sup>-1</sup>; beef peptone: 2.5 g.L<sup>-1</sup>; tryptone: 2.5 g.L<sup>-1</sup>; NaH<sub>2</sub>PO<sub>4</sub>.2H<sub>2</sub>O: 0.5 g.L<sup>-1</sup>; MgCl<sub>2</sub>: 0.05 g.L<sup>-1</sup>; MnSO<sub>4</sub>.H<sub>2</sub>O: 0.01 g.L<sup>-1</sup>; KCl: 0.15 g.L<sup>-1</sup>; CaCl<sub>2</sub>: 0.13 g.L<sup>-1</sup>; Tween 80: 1 mL.L<sup>-1</sup>, (15) adjusted to pH

5.0 and incubated at 30°C. The cultures were performed in the same conditions except the pH was adjusted to pH 3.2. Cultures were inoculated to reach a cell concentration of  $10^6$  cells.mL<sup>-1</sup>.

*Staphylococcus aureus* RN4220 (Micalis Institute) was cultivated in Brain Heart Infusion (BHI) (Biokar, Allonne, France) at 37°C. A pre-culture of 7 h was carried out before transferring 1 ml to 50 mL of culture medium.

All of the bacteria were stored in a cryotube at -80°C: an aliquot of 1.5mL of 7-h-old preculture (48-h-old preculture for *Oenococcus oeni*) was poured in the cryotube with 10% glycerol (v/v).

## 2.2. Viable cell counts

The enumeration of viable cells was performed by FCM on the basis of viability staining by cFDA. A quantity of 100 µL of culture containing approximately  $10^7$  cells.mL<sup>-1</sup> was added to 900 µL of McIlvaine's buffer [citric acid (100 mmol (Sigma Aldrich, Saint Quentin Fallavier, France), disodium hydrogen phosphate dihydrate (Na<sub>2</sub>HPO<sub>4</sub> 200 mmol, (ThermoFisher, Strasbourg, France))] adjusted to pH4 for yeast and 7.3 for bacteria. A total of 10 µL of Chemchrome V8 (Bio-Mérieux, Craponne, France) diluted to 1/10 (v/v) in acetone (Sigma Aldrich, Saint Quentin Fallavier, France) was added, and the suspension was incubated for 10 min at 40°C before flow analysis.

## 2.3. Cytometric descriptor of vitality

A quantity of 200 µL of culture containing approximately  $2.10^7$  cells.mL<sup>-1</sup> was added to 1.8 mL of McIlvaine's buffer adjusted to pH 4 for yeast and 7.3 for bacteria, and incubated with 10 µL of Chemchrome V8 (Bio-Mérieux, Craponne, France) for 10 min at 40°C.

The stained sample was centrifuged (14500 g, 90 s), and the pellet was resuspended in 2 mL of McIlvaine's buffer at pH4 for yeast or 7.3 for bacteria. One milliliter was immediately analyzed by FCM to measure the fluorescence intensity FI<sub>0</sub>. The second milliliter was incubated for 15 min (except for the development) at 40°C to promote the efflux of cF, centrifuged, and the pellet was resuspended

in McIlvaine's buffer (pH 4 or 7.3) before FCM analysis to measure the fluorescence intensity  $FI_{15}$ . Measures of fluorescence intensity  $FI_0$  and  $FI_t$  were performed on a normalized histogram containing 10,000 cells. The efflux percentage was calculated as follows:  $\Delta FI_{15} = 100(FI_0 - FI_{15})/FI_0$ .

The development of the method was performed with *S. cerevisiae*. For that, the incubation time of the second milliliter to promote the efflux of cF, was 30 min or 50 min, and the calculation of the efflux percentage ( $\Delta FI_t = 100(FI_0 - FI_t)/FI_0$ ) was compared to  $\Delta FI_{15}$ . For one experiment during the development method, yeast cells (*S. cerevisiae*) were centrifuged and the pellet was placed in osmosed water from 1.5 h to 24 h. The descriptor  $\Delta FI_{15}$  was directly measured after this time on one aliquot, and on a second aliquot after re-suspension in a glucose solution ( $1.8 \text{ g.L}^{-1}$  in osmosed water) for 20 minutes.

#### 2.4. Flow cytometry analyses

Flow cytometry analyses was performed with a CyFlow Space cytometer (Sysmex-Partec, Villepinte, France) equipped with a solid blue laser, emitting at 488 nm, and four optic filters: a forward-angle light scatter (FSC) combined with a diode collector, a side-angle light scatter (SSC) and two fluorescence signals collected with photomultiplier tubes, a 530-nm band-pass filter (515–545) to collect the green fluorescence (FL1 channel) and a 630-nm-long pass filter to collect the red fluorescence (FL2 channel). The signal analysis was performed using logarithmic gains and specific detector settings, adjusted on a sample of unstained cells (first decade for FL1), in order to eliminate cellular autofluorescence. Gating on FSC/SSC was used to discriminate bacteria or yeast cells from the background (R1 region). Data were collected and analyzed with FloMax software (SYSMEX-PARTEC, Villepinte, France) using statistical tables that indicate numbers and percentages of stained cells determined by each detector, as well as the fluorescence intensity of each fluorescent signal and the CV% (dispersion coefficient).

## 2.5. Comparison of the vitality descriptor $\Delta FI_{15}$ and the other activity descriptors

To assess and compare the measurements of the vitality of microbial cells between the reference activity method and the cytometric descriptor, the strain (yeast or bacteria) was cultured in appropriate medium. Several samples were taken throughout the growth, from the inoculation to the stationary phase. For each sample,  $\Delta FI_{15}$  was immediately measured by FCM on one aliquot, and a second aliquot was used to measure the activity with the dedicated method described below. Since these methods require culture, the second aliquot was used as an inoculum for these cultures. The values obtained with the two methods were compared.

### 2.5.1. Energy charge measurement

The energy charge measurement was performed on three bacterial strains: *Lactococcus lactis* SK11, *Oenococcus oeni* MC1 and *Staphylococcus aureus* RN4220. For each strain, seven to ten samples were taken during the culture in the appropriate medium.

For the extraction of nucleotides, 800  $\mu\text{L}$  of DMSO and 100  $\mu\text{L}$  of 10 mM EDTA were added to 100  $\mu\text{L}$  of cell suspension precisely adjusted to  $10^6$  cells. $\text{mL}^{-1}$ , mixed for 1 min and then centrifuged (14500 g, 5 min). A quantity of 900  $\mu\text{L}$  of supernatant was added to 4 mL of 20 mM Tris buffer and stored at  $-20^\circ\text{C}$  before analysis. For the measurement of nucleotide acids, ADP and AMP were converted to ATP according to (16). ATP concentration was measured with an ATP meter (MicroLumat LB 96PEG&G Berthold, Germany). The energy charge was calculated as the ratio:  $EC = (\text{ATP} + 1/2 \text{ADP}) / (\text{ATP} + \text{ADP} + \text{AMP})$ . Each data point was the mean of at least three independent cultures.

### 2.5.2. Lag time of regrowth measurement

Lag phase time for a given number of viable microbial cells depends on the activity of these cells. The shorter the lag phase time is, the higher the activity of cells will be. For this assessment, the continuous



measurement of OD at 600 nm was performed with the Bioscreen system (Bioscreen 2400620; Labsystems, Cergy Pontoise, France). This measurement was performed on two bacteria: *L. lactis* SK11 and *S. aureus* RN4220. Aliquots of  $1.10^6$  viable cells.mL<sup>-1</sup> (counted by FCM after cFDA staining) were inoculated in wells on a microplate in 200 µL of culture medium dedicated to the bacterial strain and maintained at a set temperature. During growth of *L. lactis* SK11, the microplate was shaken (by vibration) for 30 seconds at medium intensity before the OD measurement. For *S. aureus*, which prefers aerobiosis, the microplate was shaken 30 seconds per minute at 37°C. OD was analyzed and registered every 15 minutes by the software (Growth Curves for microbiology reader Bioscreen C, Biolink 5186060A; Labsystems, Cergy Pontoise, France). Five replicates of each sample were poured on the microplate (five wells). Ten wells were filled with the sterile medium without a microbial sample to have a control of potential contamination. For one culture, the result of the OD of a sample during growth was calculated as the mean of the OD of the five replicates of the sample, minus the mean of the five replicates at time zero (i.e. corrected OD). 7 to 10 samples (one per hour) were taken throughout the culture. The lag phase was the time necessary to reach a corrected OD equal to 0.1. Each data point was the mean of at least three independent cultures.

### 2.5.3. Lag time of the substrate consumption measurement

The continuous measurement of OD with Bioscreen was not possible for *Oenococcus oeni* because the growth is so slow. Activity was determined by the lag phase of the malolactic fermentation, i.e., the beginning of the substrate (malic acid) consumption. A culture of *O. oeni* in FT80 medium (100 mL), pH 3.2, was performed, and  $\Delta FI_{15}$  was measured at different times. At each these times, aliquots of  $10^8$  *O. oeni* viable cells, counted by FCM after syto9/PI staining (17), were taken from this culture, inoculated in 100 mL of FT80 medium, pH 3.2, and incubated at 30°C. Malic acid was monitored in these sub-cultures once a day (until the beginning of its consumption) using the enzymatic method (Enzytec, Biopharm, Saint Dizier au Mont d'Or, France). The malolactic fermentation lag time was

calculated as the time where the initial malic acid concentration ( $5\text{g}\cdot\text{L}^{-1}$ ) remained constant, before the beginning of its consumption. The shorter the lag time was, the greater the activity of *O. oeni* cells was (18). Each data point was the mean of two independent cultures.

#### 2.5.4. Acidifying activity measurement

For acid-producing bacteria, activity assessment was provided by measuring of the acidifying activity, which is done with the CinAc system (Ysebaert, Frépillon, France) (19). The flasks containing milk medium were placed in a water bath at  $30^{\circ}\text{C}$  and inoculated into  $10^6$  cells. $\text{mL}^{-1}$  (counted by FCM after cFDA staining). The pH is continuously measured, thus allowing the assessment of the acidifying activity by the determination of the time ( $\text{tpH}_x$ ) necessary to reach a given pH (20). Therefore, the smaller the  $\text{tpH}_x$  value (min) is, the higher the acidifying activity will be.

We measured the acidifying activity of nine samples of cells of *L. lactis* SK11 taken throughout a culture in M17-lactose at  $30^{\circ}\text{C}$ . We measured the time (h) to reach  $\text{pH}=5$  ( $\text{tpH}5$ ).  $\Delta\text{FI}_{15}$  was measured on each aliquot of the nine samples. Each data point was the mean of three independent cultures.

#### 2.5.5. Gazogen activity measurement

The activity of fermenting yeasts can be measured by the  $\text{CO}_2$  production rate in standard conditions during a culture. In the baking industry, this activity of  $\text{CO}_2$  production rate, also known as the fermentative strength of yeasts, was determined by measuring the pressure increase due to the expansion of the volume of a dough in standard conditions (temperature, time, flour and yeast quantity) according to the method referred to as the “fermentometer of Burrows” (21). For wine yeasts, the  $\text{CO}_2$  production rate was measured by weight loss (22) or by the volume of  $\text{CO}_2$  produced. We used this last method with a new device, the YAMS (Yeast Activity Measurement System), designed and developed by the SayFood unit (AgroParisTech-INRAE, Grignon, France). The flask containing 100 ml of yeast culture, maintained at  $25^{\circ}\text{C}$  with shaking at 100 rpm, is connected to a gas counter

(Ritter MilliGascounter type MGC-1, SERV'Instrumentation, Irigny, France). The measurement cell consists of two measuring chambers immersed in a solution of HCl (1.8%), which are successively filled with the gas from the flask. When a measuring chamber is filled, the buoyancy of this chamber causes the cell to tip, triggering a pulse by means of a permanent magnet. The second measuring chamber begins to fill and the first one is emptied at the same time. A pulse corresponds to 3 ml of gas. The system includes ten channels that can be sequentially measured before coming back to the first channel. The set of recorded points makes it possible to draw the CO<sub>2</sub> production curves for each of the ten channels. Using a linear regression on the curves, we can calculate the time necessary to obtain a given amount of CO<sub>2</sub> (from 1 g to 5 g in our case) and to calculate the lag time as the time before the beginning of the increase of the curve of CO<sub>2</sub> production.

To compare the vitality of yeast cells with  $\Delta FI_{15}$  and lag time fermentation, we used the yeast L2056, cultivated in AF medium (glucose 180g.L<sup>-1</sup>). Samples were taken throughout a culture, at 3 h, 5 h, 16h, 24 h, 40 h and 72h.  $\Delta FI_{15}$  was measured on one aliquot, and three flasks containing 100 mL of AF medium was inoculated with another aliquot to reach 10<sup>7</sup>.mL<sup>-1</sup> (adjusted by FCM after cFDA staining), and connected to the YAMS. CO<sub>2</sub> production was recorded over 24 h. The lag time (mean of the three flasks) was calculated and correlated with the  $\Delta FI_{15}$  value of each inoculum. Each data point was the mean of three independent cultures.

#### *2.5.6. Respiratory activity measurement*

For non-fermenting yeasts, the CO<sub>2</sub> production rate cannot be used, and it is therefore the respiratory activity that must be assessed. The specific oxygen consumption rate was determined in a 7-ml glass reaction chamber (Veral, Colombes, France) (23), thermostated at 30°C by water circulation. The homogeneity of the cell suspension in the reaction chamber was ensured by magnetic agitation. The concentration of dissolved oxygen in the suspension was measured by an oxygen electrode (Mettler Toledo, InPro 600 series O<sub>2</sub> sensors; Mettler, Paris, France) connected to a unity of regulation (Inceltec

LH-SGI, Toulouse, France), which was linked to data collection software. The oxygen electrode was calibrated in the McIlvaine buffer, pH 7.3, to 0% and 100% air saturation, by blowing bubbles of nitrogen and air, respectively. Before analysis, 6.5 mL of the McIlvaine buffer containing  $1.8\text{g.L}^{-1}$  of glucose was placed in the reaction chamber, and the  $P_{O_2}$  was adjusted to 100% (blowing air bubbles during approximately 15 min). Measurements of  $\Delta FI_{15}$  and the time necessary to reach  $P_{O_2}=50\%$  were determined on samples taken throughout a culture of *Yarrowia lipolytica* (samples at 2h, 3h, 5h, 7h, 16h and 24h). A sample was taken, centrifuged, ( $14500\text{ g}$ , 90s), washed and resuspended in the McIlvaine buffer (pH 7.3) at a concentration adjusted to  $1.4 \cdot 10^8$  viable cells. $\text{mL}^{-1}$  (counted by FCM after cFDA staining). A quantity of 500  $\mu\text{L}$  of this cell suspension (containing  $7 \cdot 10^7$  viable cells) was poured into the reaction chamber (final concentration in the reaction chamber:  $1 \cdot 10^7$  viable cells. $\text{mL}^{-1}$ ). The analysis began when cell suspension was added in the reaction chamber. The decrease of the  $P_{O_2}$  was monitored and recorded. The results were expressed in time (min) to reach  $P_{O_2}=50\%$ . Two replicates were performed for each sample.

#### 2.5.7. Fermentation activity measurement

All the previous assays allowed to compare  $\Delta FI_{15}$  with the lag time of regrowth or the start up time of metabolisms. This last experiment aimed to compare the vitality descriptor with the activity during fermentation. Alcoholic fermentation was performed with the yeast Ethanol Red® in AF medium ( $200\text{ g.L}^{-1}$  of glucose) in a 2-L bioreactor. Temperature and pH were regulated to  $36^\circ\text{C}$  and  $\text{pH}=3.2$  (addition of 2N NaOH), respectively. Ethanol Red® yeast cells were inoculated to reach  $10^6$  cells. $\text{mL}^{-1}$  in the bioreactor, from a pre-culture of 24 h. Throughout the culture, viable yeast cells were counted every two hours by FCM after cFDA staining. Ethanol concentration was assessed by GC Head-Space (Agilent Technologies Hewlett Packard, Courtaboeuf Les Ulis, France) equipped with a capillary HP-INNOWax polyethylene glycol (PEG) column ( $30\text{ m} \times 530\text{ }\mu\text{m} \times 1.00\text{ }\mu\text{m}$ ) (operating conditions: He flow:  $8.4\text{ mL/min}$ ; injection temperature:  $250^\circ\text{C}$ ; detector temperature:

240°C; and oven temperature: 75°C).  $\Delta FI_{15}$  measurements were performed on each sample. On the basis of the results of ethanol concentration and cell concentration, we were able to calculate the ethanol production rate and the specific ethanol production rate throughout the fermentation. All the data were the mean of three fermentations.

### 3 - RESULTS

#### 3.1. Development of the cytometric descriptor to assess the vitality of microbial cells

##### 3.1.1. Development of the cytometric descriptor with *S. cerevisiae* L2056

The first development concerned the wine yeast *S. cerevisiae* L2056. Figure 1a shows the histograms of the fluorescent cells after 0, 15, 30 and 50 min of incubation after staining. The fluorescence intensity  $FI_t$  decreased when the incubation time  $t$  increased because of the cF efflux from the yeast cells. According to the age of culture, these effluxes were quantified with the descriptor  $\Delta FI_t$ : the efflux was faster with young cells than with older ones (Figure 1b). For each culture age, the difference between  $\Delta FI_{15}$ ,  $\Delta FI_{30}$  and  $\Delta FI_{50}$  was small, except for the oldest cells. Moreover, the difference between two ages of culture was slightly higher for  $\Delta FI_{15}$  than for the others:  $\Delta FI_{15}(6h) - \Delta FI_{15}(11h) > \Delta FI_{30}(6h) - \Delta FI_{30}(11h) > \Delta FI_{50}(6h) - \Delta FI_{50}(11h)$ , (Figure 1b). Since the objective was to develop a rapid descriptor to monitor the variation of the efflux out of the cells throughout their growth,  $\Delta FI_{15}$  was chosen since it was more discriminating and rapid to measure.

Figure 1c presents the  $\Delta FI_{15}$  values of yeast cells sampled at 24 h of culture, corresponding to  $\Delta FI_{15}$  time 0, and for the same cells after various durations of storage in water: the  $\Delta FI_{15}$  of yeast cells after water starvation tended to zero. When yeast cells were resuspended in glucose solution before staining, the  $\Delta FI_{15}$  values were restored, confirming that the efflux of cF after staining with cFDA was energy-dependent, as already shown by other authors in various microbial models (14)(13).

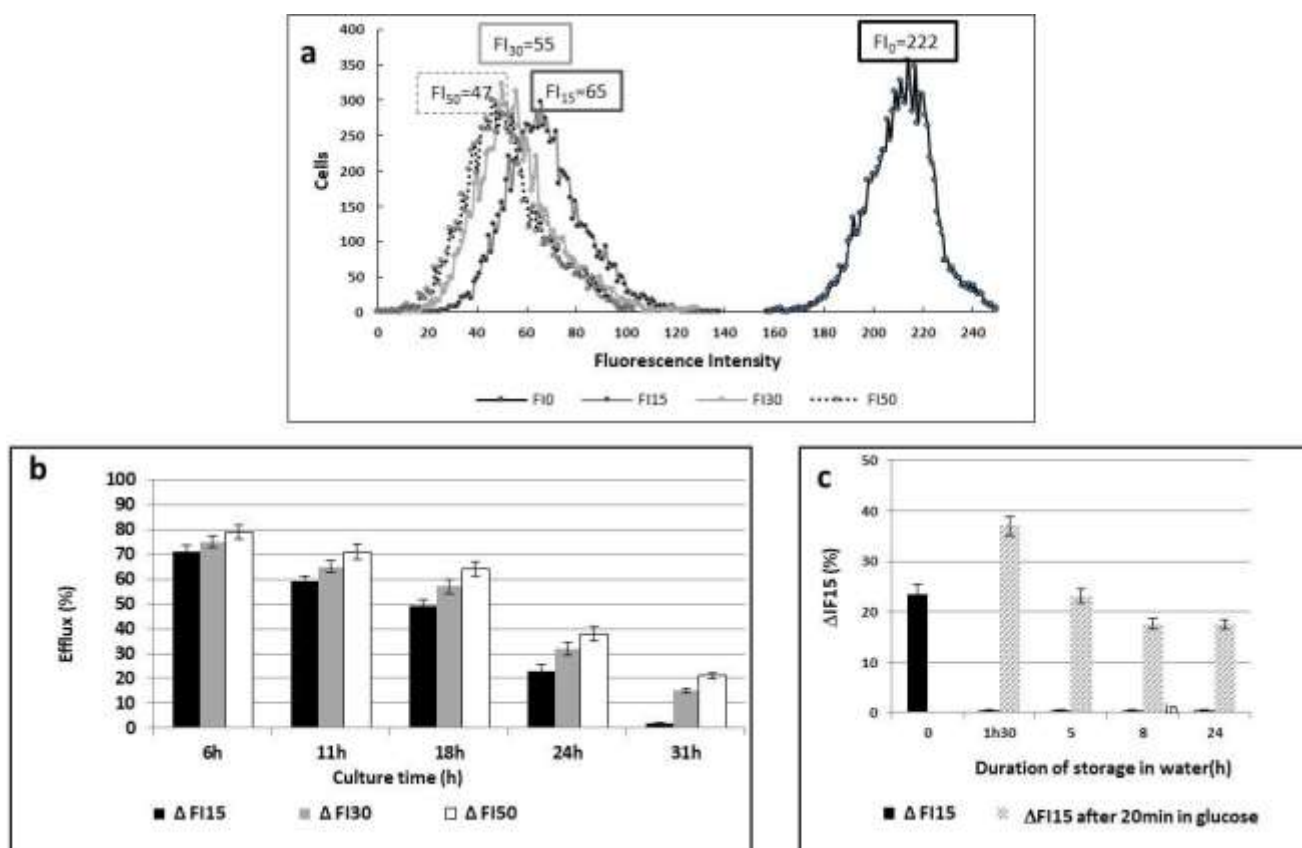


Figure 1

### Development of the vitality descriptor for *S. cerevisiae* L2056

Demonstration of the cF efflux (a);

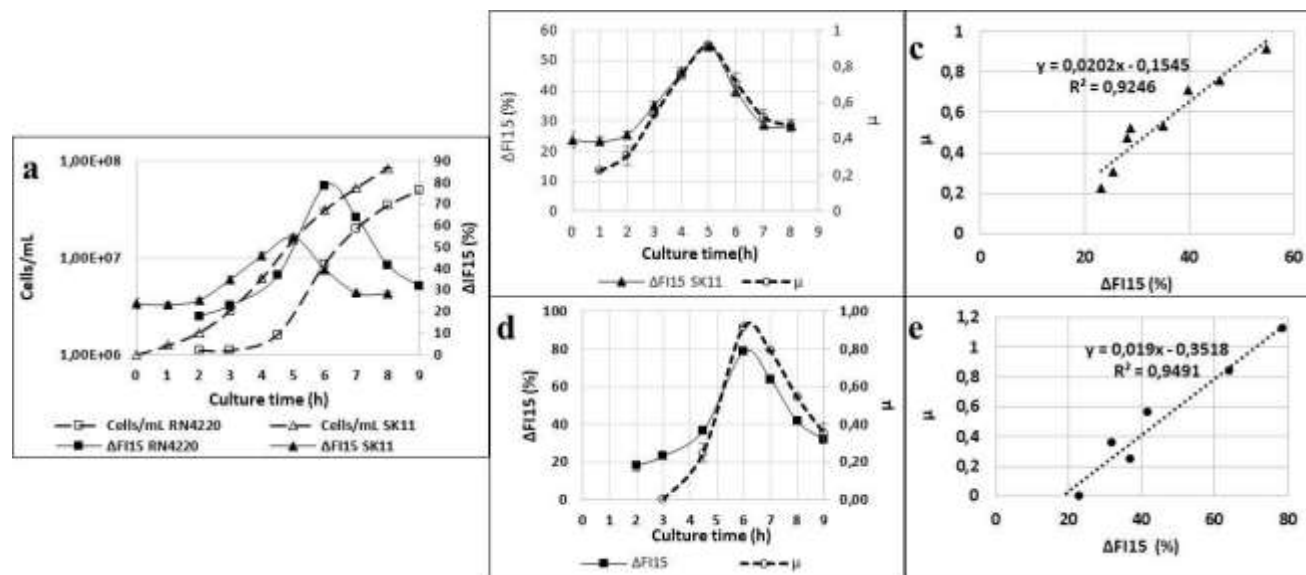
Quantification of the cF efflux depending on the incubation time (15, 30, and 50min) and the cell age (6, 11, 18, 24 and 31h) (b);

Confirmation of the energy-dependence of the efflux (cell age=24h) (c)

#### 3.1.2. Development of the cytometric descriptor with bacteria

The method developed with *S. cerevisiae* should be validated with bacteria. Throughout the growth of the lactic acid bacterium *Lactococcus lactis* ssp. *cremoris* SK11 and the bacterium *S. aureus* RN4220, the descriptor  $\Delta FI_{15}$  was measured with the same protocol as for yeast. The  $\Delta FI_{15}$  values increased at the beginning of the exponential growth and then decreased from the middle of the exponential phase of growth to the onset of the stationary phase (Fig. 2a). This change was superimposed on the evolution of the specific growth rate  $\mu$  (Fig. 2 b and d) with a coefficient of determination ( $R^2$ ) of the linear correlation equal to 0.924 for *L. lactis* SK11 (Fig. 2 c) and 0.949 for

*S. aureus* RN4220 (Fig. 2e). Thus we validated that the method developed with yeast cells for the cytometric descriptor  $\Delta FI_{15}$  can be used for bacteria.



**Figure 2**

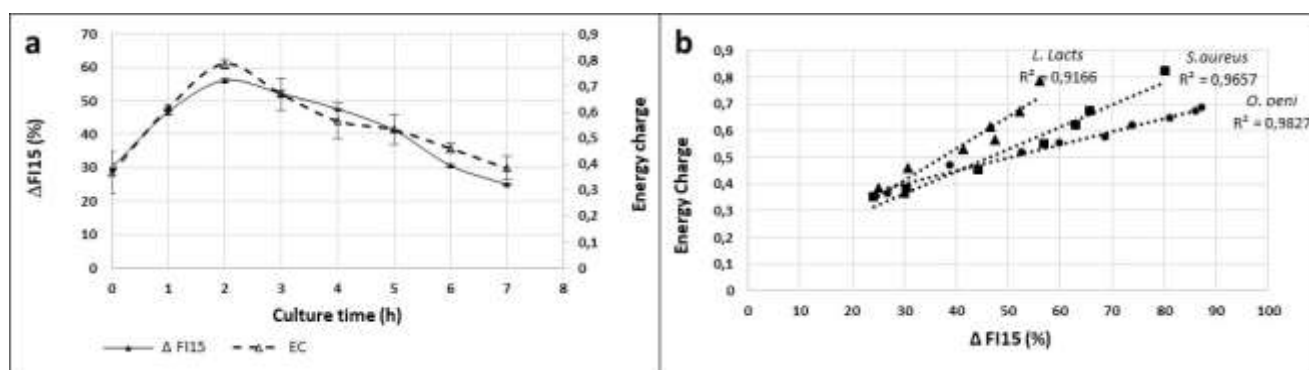
**Development of the vitality descriptor for bacteria measured by flow cytometry**

Growth curve of *L. lactis* SK11 and *S. aureus* RN4220 and respective vitality descriptor  $\Delta FI_{15}$  (a); Evolution of vitality descriptor  $\Delta FI_{15}$  and specific growth rate of *L. lactis* SK11 (b), *S. aureus* RN4220 (d), and respective linear regression curves (c, e).

**3.2. Comparison of the vitality descriptor  $\Delta FI_{15}$  with the commonly used descriptors of microbial activity**

**3.2.1.  $\Delta FI_{15}$  and energy charge**

Changes in the energy charge of bacterial cells throughout their culture were measured and compared to the variations of the descriptor  $\Delta FI_{15}$ . As displayed in figure 3, the evolution of the energy charge (EC) of *L. lactis* SK11 throughout its growth showed similar trends to that of  $\Delta FI_{15}$  (determination coefficient of 0.918, see Fig. 3b). This was similarly observed for all the other studied bacteria, namely *O. oeni* MC1 ( $R^2 = 0.981$ , Fig. 3b) and *S. aureus* RN4220 ( $R^2 = 0.965$ ).



**Figure 3**

**Correlation between the energy charge and the cytometric descriptor  $\Delta FI_{15}$**

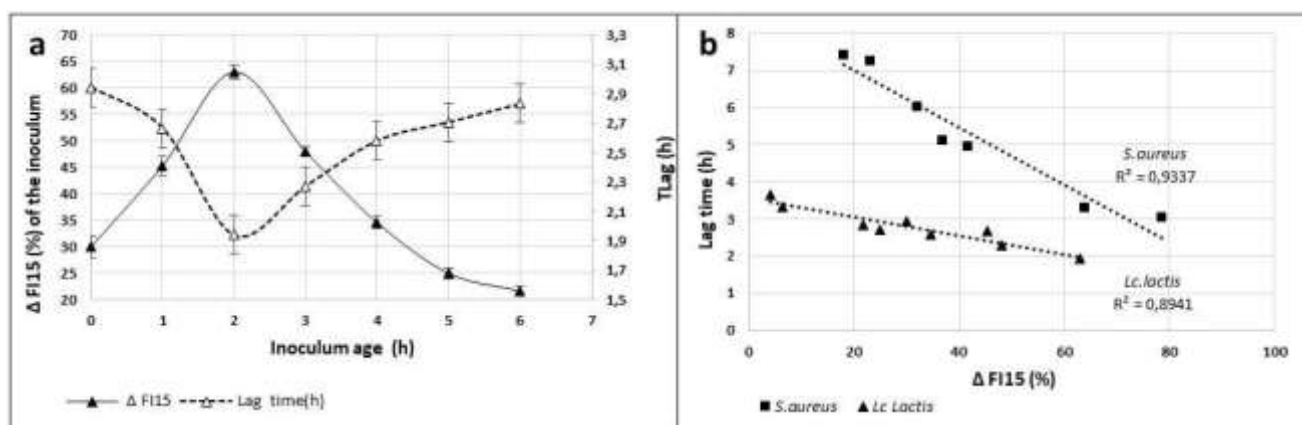
Energy charge (EC) and  $\Delta FI_{15}$  changes throughout a culture of *L. Lactis* SK11 (a);  
Linear relation between the EC and the  $\Delta FI_{15}$  descriptor for three different bacterial species (b)

**3.2.3.  $\Delta FI_{15}$  and lag time of regrowth**

The measurements of the lag time before growth were performed on the bacteria *L. lactis* SK11 and *S. aureus* RN4220 throughout a 10-h culture along with sampling and inoculation of the microplate wells every hour. The  $\Delta FI_{15}$  measurements were assessed on the same samples, one aliquot for  $\Delta FI_{15}$ , and one aliquot for the inoculum of the microplate.

For both bacteria, the lag phase duration decreased for the inocula sampled at the beginning of the exponential growth phase, then reached a minimum for the 2-h-old inoculum, and finally increased for all the inocula sampled thereafter until the beginning of the stationary growth phase (6 h). Figure 4a shows that  $\Delta FI_{15}$  measured on each of these inocula of *L. lactis* SK11 increased to a maximum for the 2-h-old inoculum and then decreased: the shorter the lag phase was, the higher the value of  $\Delta FI_{15}$  was. Fig. 4b shows the linear correlation coefficients between the lag phase duration and the  $\Delta FI_{15}$  for the two tested bacteria.





**Figure 4**

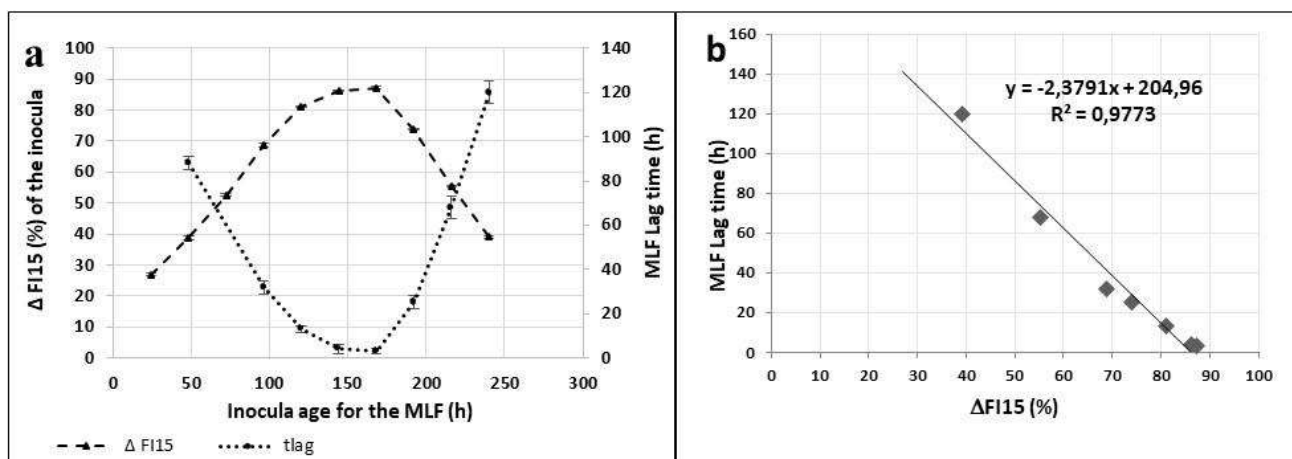
**Correlation between the duration of the lag phase of re-growth and the cytometric descriptor  $\Delta FI_{15}$**

Evolution of the lag phase duration of re-growth and the values of  $\Delta FI_{15}$  of samples taken throughout a culture of *L. lactis* SK11 (a);

Correlation between lag time and  $\Delta FI_{15}$  for *L. lactis* SK11 and *S. aureus* RN4220 (b).

**3.2.4.  $\Delta FI_{15}$  and Lag time of substrate consumption**

The growth of *O. oeni* in FT80, pH3.2, was slow since the fermentation time was 250 h. For inocula taken from a culture aged between 50 and 150 h,  $\Delta FI_{15}$  increased from 28% to 88% and then decreased to 40% for an inoculum sampled at 250h of culture (Fig5a). The duration of the lag phase of the malolactic fermentation of the cultures inoculated with *O. oeni* cells characterized by these different values of  $\Delta FI_{15}$  followed an inverse evolution: the lag phase was very long for the culture inoculated with cells showing weaker  $\Delta FI_{15}$  cells, (inoculum age between 50 and 100 h), increasingly short with the cells taken at around 150 h of culture showing the highest  $\Delta FI_{15}$ , and then increased up to 120 h for cells of the oldest inoculum (Fig. 5a). The shorter the latency period of malic acid consumption was, the greater the cell activity and the higher the value of the descriptor  $\Delta FI_{15}$  were. The determination coefficient of the linear correlation between  $\Delta FI_{15}$  and the latency period of malic acid consumption was 0.977 (Fig. 5b).

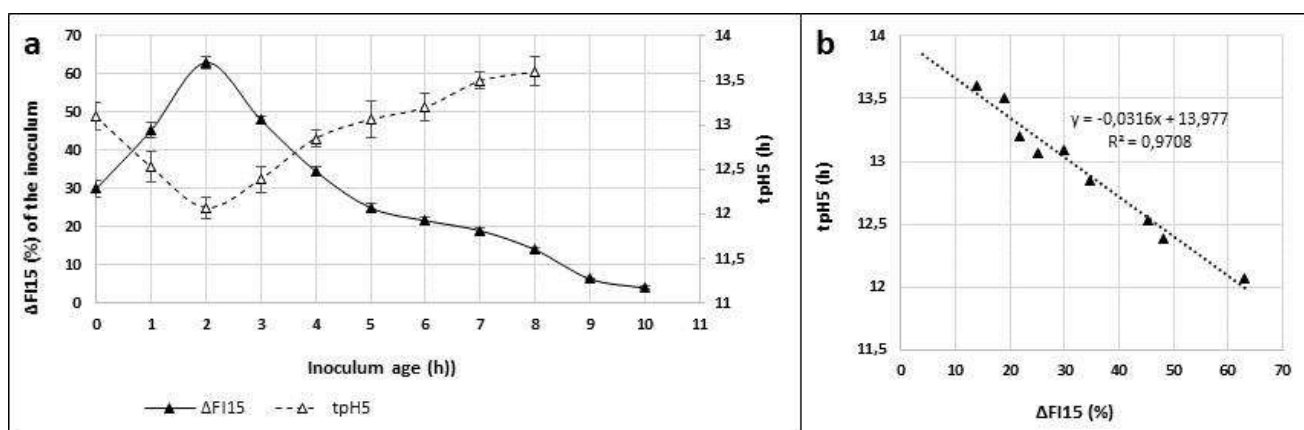


**Figure 5**  
**Correlation between the duration of the lag phase of malolactic fermentation by *Oenococcus oeni* MC1 and the cytometric descriptor  $\Delta FI_{15}$**

Evolution of the Malolactic Fermentation (MLF) lag time and  $\Delta FI_{15}$  values of bacterial samples. (a) correlation curve (b)

### 3.2.5. $\Delta FI_{15}$ and acidifying activity

The acidifying activity of lactic acid bacteria can be measured by the time needed to reach a given pH value. A difference of about 1,5h for the time to reach a pH value of 5 was observed between the fastest and the slowest aliquots of *L. lactis* SK11 cells. As shown in Fig. 6a, the cells sampled at the beginning and at the end of the culture required 13.5-14 h to reach a pH of 5, whereas those sampled after 2 h of culture required only 12 h to reach the same pH value. The acidifying activity of bacterial cells was maximum after 2 h of culture, and decreased after this time. The curve of the cytometric descriptor  $\Delta FI_{15}$  showed the inverse evolution: it was maximum for the cells sampled at 2 h of culture and lower before and after this point.  $\Delta FI_{15}$  was negatively correlated to the acidifying activity, with a determination coefficient of 0.97 (Fig. 6b).



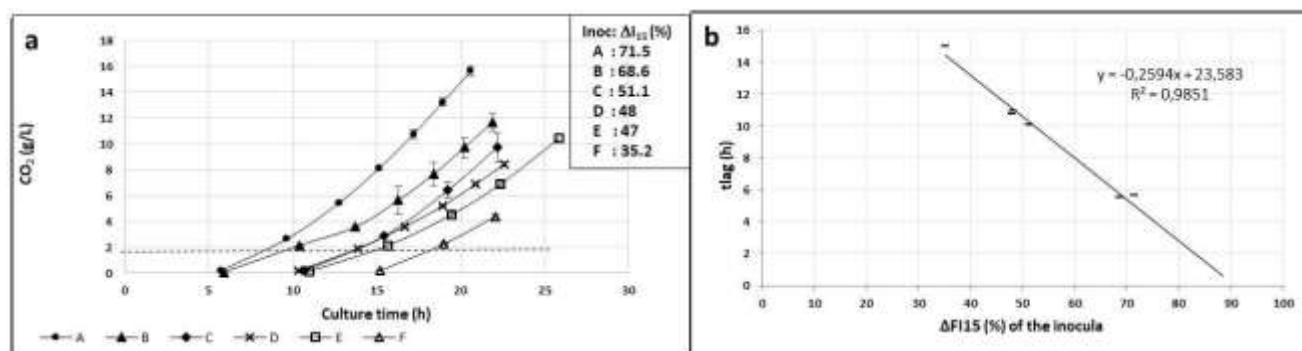
**Figure 6**

**Correlation between the acidifying activity of lactic acid bacteria and the cytometric descriptor Time to reach a pH value of 5.0 ( $t_{pH5}$ ) of different samples of *L. lactis* SK11 taken throughout a culture used as inoculum and corresponding  $\Delta FI_{15}$  values of each inoculum (a)**

**Correlation between  $t_{pH5}$  and  $\Delta FI_{15}$  (b).**

### 3.2.6. $\Delta FI_{15}$ and gazogen activity (fermenting yeast)

The YAMS made it possible to record the  $CO_2$  production rate of the cultures inoculated with the samples taken throughout a previous culture (sampling time of A: 3h, B: 5h, C: 16h, D: 24h, E: 40h, and F: 72h; Fig. 7a). The  $\Delta FI_{15}$  values of these samples (= inocula of the cultures) changed from 71.5% to 35%. Depending on the samples, the time to produce 2 g of  $CO_2$  (dashed line on Fig. 7a) changed from 8 h to 18 h. The lag phase (t<sub>lag</sub>) (i.e., time before the beginning of the increase of the curve of  $CO_2$  production: before the lag time, the  $CO_2$  production was too low to be detected) then varied from 5.5 h to 15 h. The coefficient of determination ( $R^2$ ) of the linear correlation between the t<sub>lag</sub> and  $\Delta FI_{15}$  was equal to 0.98. (Fig. 7b).



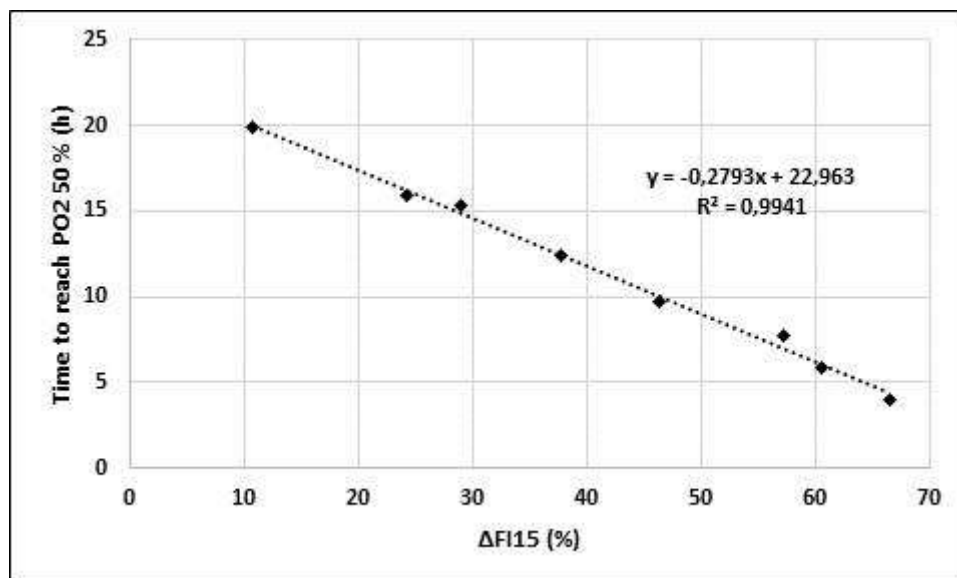
**Figure 7**

**Correlation between the CO<sub>2</sub> production rate of the fermenting yeast *S. cerevisiae* L2056 and ΔFI<sub>15</sub>**

CO<sub>2</sub> production rate of six cultures with various ΔFI<sub>15</sub> values of the inocula (a);  
Correlation between the lag time of CO<sub>2</sub> production and the ΔFI<sub>15</sub> values of the inocula (b).

### 3.2.7. ΔFI<sub>15</sub> and Respiratory activity

For the strictly aerobic yeast *Yarrowia lipolytica*, metabolic activity was measured by the oxygen consumption rate. As the experimental device corresponds to a small volume and needs a high cell concentration ( $10^7 \text{ mL}^{-1}$ ) to be able to get a measurement, the time to reach 50% of partial dissolved oxygen pressure ( $P_{O_2}$ ) was evaluated in minutes. It varied from 5 min to 20 min depending of the age of the inoculum. The vitality descriptor ΔFI<sub>15</sub> changed from 66% to 10%. The coefficient of determination of the linear correlation between the vitality descriptor ΔFI<sub>15</sub> and the time to reach 50% of  $P_{O_2}$  (starting to 100% at the beginning of the assay) was equal to 0.99 (Fig.8).

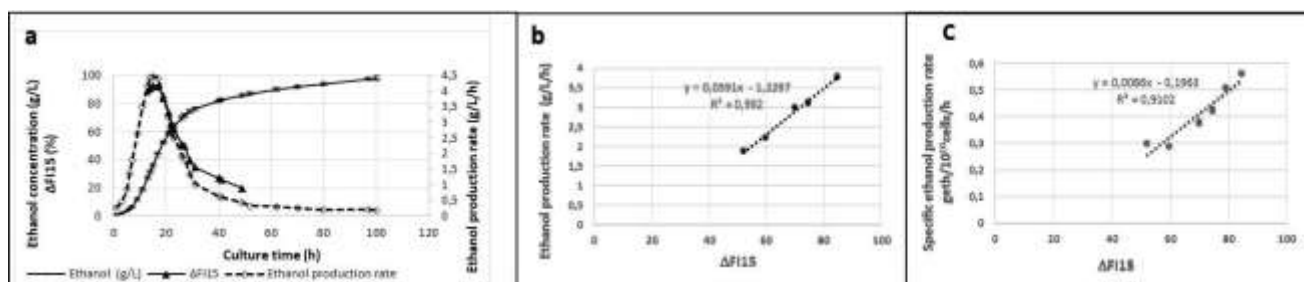


**Figure 8**

**Correlation between the time to reach a partial pressure of dissolved oxygen ( $P_{O_2}$ ) of 50% (starting from 100%) during cultures of *Yarrowia lipolytica* and  $\Delta FI_{15}$  values of the samples**

### 3.2.8. $\Delta FI_{15}$ and fermenting activity

Throughout the alcoholic fermentation performed with the yeast Ethanol Red®, the ethanol concentration increased from 0 to 98 g.L<sup>-1</sup> (Fig9a). The cell concentration changed from 10<sup>6</sup> cells.mL<sup>-1</sup> at the beginning of the culture to 7.10<sup>7</sup> cells.mL<sup>-1</sup> at 40 g.L<sup>-1</sup> of ethanol, 8.10<sup>7</sup>.mL<sup>-1</sup> at 70 g.L<sup>-1</sup> of ethanol, and remained stable until the end of fermentation. The vitality of yeast cells measured by  $\Delta FI_{15}$  decreased when the ethanol concentrations increased. The change in the vitality followed the same profile as the ethanol production rate (Fig9a). The coefficients correlation between  $\Delta FI_{15}$  and ethanol production rate, on the one hand, and between  $\Delta FI_{15}$  and the specific ethanol production rate, on the other hand, were linear (with coefficients of determination of 0.99 and 0.91, respectively (Fig9b, 9c). The lower R<sup>2</sup> value in the case of the correlation with the specific ethanol production rate may be explained by the accuracy of the measurement of the cellular concentration, which was worse than that of the ethanol concentration.



**Figure 9**

**Correlation between fermenting activity throughout a culture and  $\Delta FI_{15}$**

Ethanol production, ethanol production rate and  $\Delta FI_{15}$  (a);

Correlation between the ethanol production rate and  $\Delta FI_{15}$  (b);

Correlation between the specific ethanol production rate and  $\Delta FI_{15}$ (c).

#### 4 – DISCUSSION AND CONCLUSION

As reported by (13) and (14), we confirmed that after staining with cFDA cleaved by intracellular esterase, yeast cells and bacterial cells extruded cF via an energy-dependent mechanism. The aim of our study was not to clarify the details of the mechanisms, but to quantify the efflux in order to propose an easy-to-handle descriptor of cell vitality on various microorganisms. A 15-min efflux was demonstrated as the most discriminating for cells of different culture ages, and made it possible to have a relevant descriptor of microbial cell vitality that could be assessed in a short time, both for yeast and bacteria.

This study confirmed that the vitality of microorganisms measured by the proposed cytometric descriptor changes throughout a culture, following the profile of the specific growth rate and the increase and decrease in the energy charge. All the measurements for an assay were performed with samples at equal cell concentrations, but with different specific activities. This difference of a few hours may be significant in industrial processes, resulting in a more or less quick start-up of culture after inoculation. For example, in cheese-making, the starter must be able to colonize the milk and acidify it as quickly as possible in order to prevent the development of spoilage and pathogenic bacteria. When we inoculated two milk samples with *S. aureus* RN4220 ( $10^6$  cells.mL<sup>-1</sup>) and with *L. lactis* SK11 ( $10^6$  cells.mL<sup>-1</sup>), but one with *L. lactis* characterized by a high  $\Delta FI_{15}$  and the other with *L.*

*lactis* showing a lower  $\Delta FI_{15}$ , we observed that *S. aureus* grew to  $1.6 \cdot 10^7$  cells.mL<sup>-1</sup> and then dropped to  $2.9 \cdot 10^6$  cells.mL<sup>-1</sup> after 7h of culture and to  $9 \cdot 10^5$  cells.mL<sup>-1</sup> after 24 h of culture in the first sample, but grew to  $6.6 \cdot 10^7$  cells.mL<sup>-1</sup> and remained at this level after 24 h in the second sample (*S. aureus* RN4220 alone:  $9.5 \cdot 10^7$  cells.mL<sup>-1</sup> after 7h of culture) (data not shown). This proved the role of the vitality of the starter *L. lactis* SK11 independently of the viable count. In addition, a weak vitality of yeasts may explain why, in some cases, wine fermentation remains slow (sluggish fermentation) (24).

To validate the pertinence of the cytometric vitality descriptor, we compared the  $\Delta FI_{15}$  values with the measurements performed with the reference methods for activity assessment suitable for each dedicated metabolism. All  $\Delta FI_{15}$  measurements were well correlated with high determination coefficient to each specific activity tested, both for yeasts and bacteria, regardless of the metabolism, i.e., acidifying activity, CO<sub>2</sub> production or oxygen consumption, lag time of regrowth, substrate consumption or metabolite production.

To our knowledge, there is no other cytometric descriptor to assess the vitality of bacteria as well as yeasts. A review of methods (25) to measure yeast vitality cited the measurement of mitochondrial membrane potential by fluorescence microscopy, after application of an oxidative stress, and not on cells in cultures under natural fermentation conditions. The  $\Delta FI_{15}$  descriptor is therefore the only cytometric descriptor to assess vitality for both bacteria and yeast currently available. This rapid cytometric descriptor proposed in this study included two steps: first, the staining of the cells with cFDA for an incubation of 10 min, then the efflux of cF from the cells for 15 min after centrifugation and re-suspension in a buffer (2 min), followed by the cytometric analysis. It means a total duration of approximately 30 min to assess the cellular concentration (on the same aliquot as the fluorescence intensity  $FI_0$ ) and the microbial cell vitality thanks to the calculation of  $\Delta FI_{15}$ . This time is much shorter than the time necessary to assess the different metabolic activities with the reference methods

described above since these methods require culture and several hours (or even days). Moreover, this staining and the flow cytometric analysis are easier as no preparation of sterile media in flasks or in microplates is needed. In addition, the width of the histogram makes it possible to visualize the cellular heterogeneity among the microbial population (the narrower it is, the more homogeneous the population will be), in contrast to the measurements of metabolic activities carried out on the whole population. In conclusion, the cytometric descriptor  $\Delta FI_{15}$  is consistent with reference methods of measurement of metabolic activities, gives more information than them, but much faster and is then very useful for assessing the vitality of microorganisms before inoculation in laboratory research or throughout an industrial process.

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