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BIOTECHNOLOGY METHODS

Cytofluorometric detection of wine lactic acid bacteria: application of malolactic fermentation to the monitoring

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Abstract In this study we report for the first time a rapid, efficient and cost-effective method for the enumeration of lactic acid bacteria (LAB) in wine. Indeed, up to now, detection of LAB in wine, especially red wine, was not possible. Wines contain debris that cannot be separated from bacteria using flow cytometry (FCM). Furthermore, the dyes tested in previous reports did not allow an efficient staining of bacteria. Using FCM and a combination of BOX/PI dyes, we were able to count bacteria in wines. The study was performed in wine inoculated with *Oenococcus oeni* (10^6 CFU ml^{-1}) stained with either FDA or BOX/PI and analyzed by FCM during the malolactic fermentation (MLF). The analysis show a strong correlation between the numbers of BOX/PI-stained cells determined by FCM and the cell numbers determined by plate counts (red wine: $R^2 \geq 0.97$, white wine $R^2 \geq 0.965$). On the other hand, we found that the enumeration of *O. oeni* labeled with FDA was only possible in white wine ($R^2 \geq 0.97$). Viable yeast and LAB populations can be rapidly discriminated and quantified in simultaneous malolactic-alcoholic wine fermentations using BOX/PI and scatter parameters in a one single measurement. This rapid procedure is therefore a suitable method for monitoring *O. oeni* populations during winemaking, offers a detection limit of $<10^4$ CFU ml^{-1} and can be considered a useful method for investigating the dynamics of microbial growth in wine and applied for microbiological quality control in wineries.

Keywords Lactic acid bacteria · Malolactic fermentation · Flow cytometry · Fluorescein diacetate · Bis-oxonol

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Introduction

Wine is the result of a complex interaction between yeast, lactic acid bacteria (LAB) and must. *Oenococcus oeni* is the main species that carries out malolactic fermentation (MLF) in wine, and *Saccharomyces cerevisiae* is the main yeast species that carries out alcoholic fermentation (AF). Various bacterial genera such as *Lactobacillus*, *Pediococcus* and *Leuconostoc* can also be found in wine and can cause spoilage [1].

Real-time microbial analysis during fermentation allows winemakers to make informed production decisions. However, accurate microbial analysis of mixed populations of yeast and bacteria that are constantly changing during fermentation is a challenging task.

Several methods are available to detect and enumerate the microorganisms present in wine fermentations. Classical microbiological techniques can be used to detect LAB in wine samples, but these assays require up to 14 days to be complete because of the slow growth of some LAB such as *O. oeni*. Another disadvantage is the failure of these methods to detect viable but nonculturable organisms [2]. On the other hand, total microscopic enumeration methods are relatively fast, but are limited by operator fatigue due to prolonged microscope use and by low sensitivity [3]. Consequently, several molecular methods have been developed, but most of these methods, such as species-specific

PCR and nested PCR, require lysis of the microbial cells and isolation of DNA [4]. Species-specific target sequences can also be detected in DNA immobilized on a membrane, DNA in solution or after the DNA has been resolved on gel, using either radioactive or fluorescent oligonucleotide probes [4, 5]. Alternatively, qPCR amplification has been used to detect and identify *O. oeni* in wine [6]. For all these methods, primer design requires prior knowledge of genomic sequences, the cost of the analysis is expensive [7], and analysis times generally exceed 6 h [6].

Flow cytometry is extremely sensitive, it avoids the need for culturing or enrichment procedures, and it can be both qualitative and quantitative [3, 8, 9]. FCM offers numerous possibilities for the detection and enumeration of microorganisms in their natural habitat. It also allows the detection and discrimination of viable cultivable, viable noncultivable and nonviable organisms by using fluorescent stains or fluorogenic substrates [2, 3, 10–14]. Continuous improvements in the sensitivity and performance of FCM instruments have resulted in a wide range of applications for characterizing and detecting bacteria, yeast, fungi and even viruses [8, 13, 15–19].

FCM has been used previously to monitor microbial populations in wine fermentations and was found to be suitable for enological applications [20–23]. The method has also been validated by comparing it with other viability tests such as plating methods [22–24].

Rodriguez et al. [23] proposed to monitor yeast and bacteria simultaneously in white wine using flow cytometry with fluorescent antibodies. However, antibodies are expensive, and the efficiency of this method in red wine still needs to be demonstrated.

Malacrinò et al. [22] used FCM to directly detect yeasts and malolactic bacteria in wine using different fluorescent viability probes, such as rhodamine 123, calcein acetoxymethyl ester and fluorescein diacetate (FDA). FDA was found to be the most effective marker for labeling *O. oeni* because it generates a higher fluorescent signal than the autofluorescence produced by cells and wine debris [22]. FDA is a lipophilic, uncharged and non-fluorescent substrate for cellular esterase, which cleaves FDA inside living cells to release green fluorescent fluorescein (520-nm emission). FDA can be used to monitor cellular esterase activity and to determine the viability of cell populations. However, the fluorescein generated from FDA has been reported to leak from cells [25]. In addition, natural wine debris can interfere with *O. oeni* detection and can cause poor quantification results [22]. Furthermore, some *O. oeni* strains are not stained at all with FDA, which limits the usefulness of this probe for detecting these bacteria in wine [22].

A number of different fluorescent probes could be potentially applied to the microbial analysis of wine. The combination of a cell dye, such as bis-oxonol (BOX), and propidium iodide (PI) could be used to distinguish between intact polarized cytoplasmic membranes (CMs), intact depolarized CMs and permeabilized membrane cells.

PI is a vital red fluorescent probe (635-nm emission) that binds to DNA only when the CM is permeabilized [2, 26–28]. BOX is a lipophilic, non-toxic anionic green fluorescent (525-nm emission) stain that binds to the CM only if it is depolarized. BOX staining also provides a measure of the cellular metabolic "stress" level. One advantage of using BOX is its insensitivity to the efflux pump systems in the CM [2]. Thus, dual staining with BOX/PI can provide quantitative and qualitative information about the cell populations having intact polarized CMs, intact depolarized CMs and permeabilized membrane cells. Use of this combination of fluorescent dyes for microbial analysis of wine has not been reported previously.

The first objective of this study was to compare the efficacy of FDA staining to that of BOX/PI staining in the detection and quantification of LAB in wine. Our second objective was to develop a method for a quick and reliable quantitative FCM assay for LAB in wine. Thereafter, we applied FCM: (1) to evaluate the viability of different *O. oeni* starter ferments during winemaking, and (2) to monitor the changes in the bacterial and yeast populations during fermentation of musts, inoculated simultaneously with *S. cerevisiae* and *O. oeni*.

Materials and methods

Yeast and bacterial strains; culture media

Saccharomyces cerevisiae strain PB2585 was aerobically cultured on yeast extract-peptone-dextrose agar medium [YPD; 10 g l^{-1} yeast extract (Conda catalog no. 1702, Madrid, Spain); 20 g l^{-1} glucose (Sigma G8270, Quentin Fallavier France), 20 g l^{-1} bacterial peptone (Conda catalog no. 1616, Madrid, Spain), 20 g l^{-1} agar (Conda catalog no. 1805, Madrid, Spain)] at 28 °C. For liquid culture, yeast cells were grown in YPD broth at 28 °C.

Pediococcus damnosus LMG16740 and *O. oeni* (DIV SABO11 and DIV 7.32) were cultured on LAC medium [78 ml l^{-1} white commercial grape juice, 33 g l^{-1} yeast extract (Conda catalog no. 1702, Madrid, Spain), 0.6 ml l^{-1} Tween 80 (Sigma P4780, Steinheim Germany), 0.08 g l^{-1} $MnSO_4$ H_2O (Sigma M7634, Quentin Fallavier France)] containing 1 ml Delvocid 1 % to suppress yeast growth. The plates were incubated anaerobically at 28 °C for 10–14 days inside jars using an anaerobic atmosphere generation system (Anaerogen Kits, Oxoid Ltd). *Lactobacillus plantarum* WCFS1 was cultured at 30 °C in MRS medium (Conda, catalog no. 1215, Madrid Spain). For liquid culture, *P. damnosus* and *O. oeni* cells were grown in

LAC broth at 28 °C. *Lactobacillus plantarum* WCFS1 was precultured at 30 °C in MRS broth (Conda, catalog no. 1215, Madrid Spain).

Two commercial starters of *O. oeni*, starter I (Inoflore, Institut Oenologique de Champagne, France) and starter II (Viniflora®—Oenos, Denmark), were used according to the manufacturer's rehydration and inoculation instructions.

Musts and wines

Musts were collected from the Burgundy area at harvest. Red (Pinot noir) and white (Chardonnay) wines (pH 3.17 and 3.52, respectively) were also from the Burgundy area. For some experiments, samples of wine were filtered through a 0.22-µm-pore membrane (Sartorius). To control the sterility of filtered wine, 1 ml of wine was aseptically removed and incubated for 3 days on (YPD) agar medium at 28 °C to culture yeast or for 14 days on LAC agar medium at 28 °C in anaerobic conditions to culture bacteria as previously described.

Flow cytometry analysis

Flow cytometry samples were analyzed using a Guava EasyCyte Plus SSC4C flow cytometer (Guava Technologies, Hayward). This instrument is equipped with a 488-nm, 25-mW laser line, forward scatter (FSC, for cell size) and side scatter (SSC, for granularity) detectors; green fluorescence was collected on the FL 1 channel using a 525-nm (±30 nm) band-pass filter; red fluorescence was collected on the FL 3 channel using a 680-nm (±30 nm) band-pass filter. This instrument allows determining accurate cell numbers and population percentages, without the need for reference beads, as described by the manufacturer using only the Guava Cytosoft data acquisition and analysis software. For all analyses, a minimum of 5,000 events was acquired, and all samples were collected as logarithmic signal. Experiments were performed in duplicate and included an unlabeled sample as a control in 96-well plates. Data were analyzed using the Guava Cytosoft data acquisition and analysis software version 5.0 and FlowJo software version 7.6. Hardware compensation has been applied for adjusting the settings of the two-color analysis (BOX/PI, Table 1). In parallel, cells were enumerated by plating on YPD and LAC agar media to validate the FCM results.

Analysis of strains and wine debris by scattered light detection on a flow cytometer

Unfiltered and filtered wines, either inoculated with microorganisms or not, were analyzed on a flow cytometer by scattered light detection using forward scatter (FSC) and side scatter (SSC) using logarithmic scale. After preculture, bacteria were inoculated in wines at a density of 10^6 CFU ml^{-1} and incubated at 20 °C. Commercial starters were directly inoculated according to the manufacturer's rehydration and inoculation instructions (Fig. 1). All culture processes were performed in triplicate.

Alcoholic and/or malolactic fermentations

Fermentations were carried out in 250-ml flasks containing 200 ml of must or wine. All fermentations were performed in triplicate.

After preculture, LAB strains were inoculated in wines at a density of 10^6 CFU ml^{-1} and incubated at 20 °C. Commercial starters were directly inoculated according to the manufacturer's rehydration and inoculation instructions (Fig. 1). MLF was monitored by malic acid degradation analyzed by an enzymatic assay (OENOSENTEC L-malic acid kit, Toulouse, France) according to the manufacturer's instructions. MLF was considered to be complete when the malic acid concentration was less than 0.2 g/l.

In cases of co-inoculation, starters and *S. cerevisiae* PB2585 were simultaneously inoculated at densities of 10^6 CFU ml^{-1} in must and incubated at 20 °C (Fig. 1). AF was monitored by sugar consumption using the DNS method [29] and was considered to be complete when the sugar level was less than 1 g/l.

Detection of strains with fluorescent dyes

We used three fluorescent dyes BOX/PI and FDA to detect microorganisms by FCM. For the FDA staining procedure, 1 ml of cultured cells from red or white wine was centrifuged (13,000 rpm for 5 min at 25 °C), the pellet was rinsed twice in PBS (130 mM NaCl, Sigma S9888, Quentin Fallavier France), 5 mM NaH_2PO_4 (Sigma S2554, Quentin Fallavier France) and 5 mM Na_2HPO_4 (Sigma 255793, Quentin Fallavier France; pH 7.4). Cells were resuspended in 1 ml of FDA buffer (0.5 M Na_2HPO_4 and 0.5 M NaH_2PO_4; pH 7) to which 6 µl of FDA at 20 µM in acetone (Sigma F737, St Louis, MO, USA) was added in order to reach a final concentration of 0.12 µM, and the cells were then incubated for 15 min at room temperature in the dark before being analyzed by FCM. Samples were diluted if necessary to obtain a final concentration of 10^6 CFU ml^{-1}.

To stain cells with BOX and PI, 100 µl of cultured cells from inoculated wine or must was added to 900 µl of PBS (pH 7.2–7.5), and 4 mM EDTA (Sigma E9894, Steinheim Germany) was added to facilitate the staining of gram-negative bacteria with BOX by destabilizing the outer membrane (OM) [30]. Cell concentrations between 5×10^5 and 1×10^6 cells ml^{-1} are recommended for counting with FCM; 1 µl of 2 mM PI (final concentration

2 μM; Sigma, 81845, St Louis, MO, USA, in sterile MilliQ water) and 3 μl of 2 mM BOX (final concentration 6 μM; Invitrogen, *B-438*, USA, in DMSO, Sigma, 472301, Steinheim, Germany) were added to the resuspended cells to label dead and viable cells, respectively. The cell suspensions were incubated at room temperature for 15 min in the dark before being analyzed by FCM.

Fluorescence microscopy

Microscope slides were analyzed with an epifluorescence microscope (Leica DM LB) equipped with a 12-V, 50-W halogen lamp for transmitted light illumination, a 50-W mercury arc lamp for epifluorescence illumination, a fluorescein isothiocyanate filter set and a 100× objective lens.

Table 1 Typical cytometer settings used for analysis of bacteria species

Parameter	Setting for stain(s) and species	
	FDA	Box/PI
FSC	Log A	Log A
SSC	Log A	Log A
FL1 (525 ± 30 nm)	Log A: 524 V	Log A: 557 V
FL3 (680 ± 30 nm)	Log A: 500 V	Log A: 610 V
Threshold on FSC (FSC-Hlin)	14 units	14 units
Compensation (RED—%GRN)	NA	15,9

FSC Forward scattered light, *SSC* side scattered light, *FL1* detector for green fluorescence, *FL3* detector for red fluorescence, *NA* not npplicable, *Log A* logaritmic amplification

Results

Flow cytometer instrument settings

Before the quantification of LAB in wine, a threshold level determination is required. Unfiltered wine with or without *O. oeni* cells was analyzed and compared by flow cytometry using only the FSC and SSC parameters (logarithmic scale). This allowed setting an electronic threshold on FSC to eliminate instrument noise and background linked to wine debris smaller than bacteria (data not shown). The threshold is fixed on FSC and is applied to all the following analyses (Table 1).

Measurement of cells versus particles in white wine

In a second set of experiments, we assessed whether it was possible to differentiate *O. oeni* from white wine debris (equivalent to bacteria size) based on FSC and SSC parameters. FCM analysis demonstrated that filtered and unfiltered white wines, whether inoculated or not with LAB, were not significantly different from each other in terms of light scattering (Fig. 2). Thus, it was not possible to differentiate between LAB and white wine debris by light scattering; the same results were observed for red

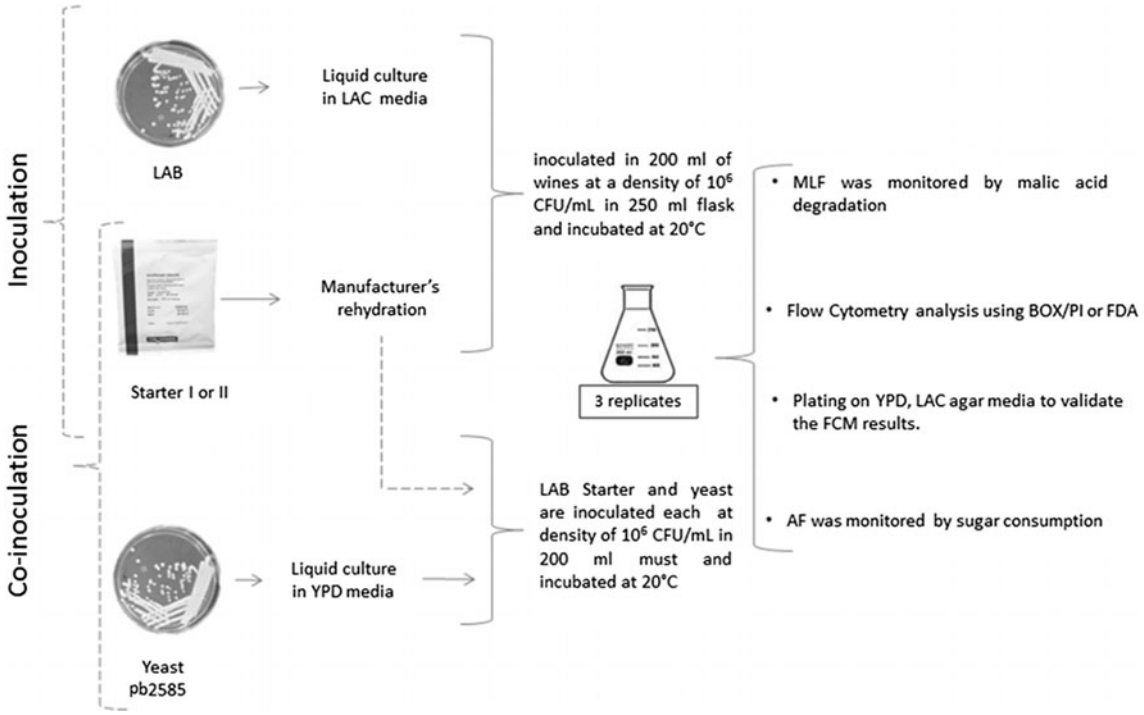

Fig. 1 Schematic summary of the experiments conducted for this study

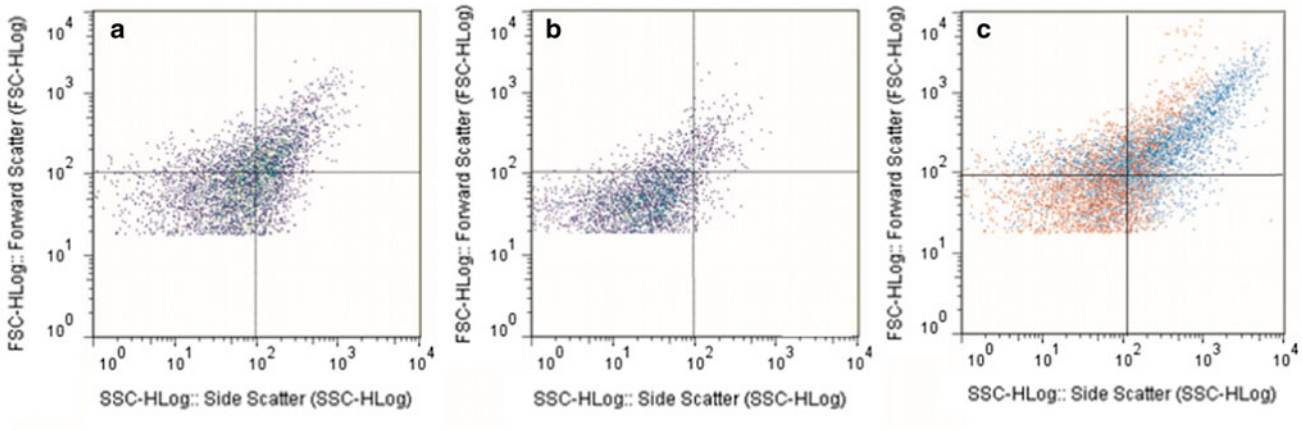

Fig. 2 Forward- (FSC-H) and side-scattering (SSC-H) results from FCM analyses of uninoculated unfiltered white wine (*Panel* **a**), uninoculated filtered red wine (*Panel* **b**), and unfiltered (*red*) and filtered (*blue*) red wine inoculated with *O. oeni* (10^6 CFU ml^{-1}) (*panel* **c**) (colour figure online)

wines (data not shown). Moreover, the autofluorescences of LAB and of wine debris were not significantly different (data not shown). We therefore investigated whether bacteria in wine could be labeled with fluorescent probes such as FDA or BOX/PI.

Bacteria staining in white wine

Analysis of *O.oeni*, inoculated in white wine by FCM using the BOX/PI, allows us to identify different subpopulations. The PI-negative cells with an intact cytoplasmic membrane (CM), which are considered to be culturable cells [2, 12, 31–34], are depicted on quadrants 3 and 4 (Fig. 3). PI-positive cells (PI positive) with a nonintact CM, which were considered to be uncultivable cells [34, 35], are depicted on quadrants 1 and 2 of Fig. 3. Quadrant 3 shows PI-negative/BOX-negative cells representing polarized cells with an intact CM, which were considered to be healthy and viable [34]. Quadrant 4 shows PI-negative/BOX-positive cells representing depolarized cells with an intact CM, which were considered to be viable but metabolically stressed (with a disrupted electron transport system across the cell membrane) cells [34].

As the wine contains different depolarizing factors such as ethanol, acidic pH (3.5), sulfites and phenol products [17, 26–28, 36–38], it was hypothesized that viable and culturable bacteria were mostly present in quadrant 4. In order to confirm our hypothesis, *O. oeni* populations during MLF in white wine inoculated with 10^6 CFU ml^{-1} were monitored by FCM using BOX/PI staining. A strong correlation between the bacteria numbers obtained by FCM [BOX/PI (quadrant 4), $R^2 \geq 0.965$] and those determined by plate counts was found (Fig. 4). This confirms that in wine most bacteria cells possess a depolarized cytoplasmic membrane. In white wine, a strong correlation also exists between the number of FDA-stained bacteria determined by FCM and plate count ($R^2 \geq 0.923$, Fig. 4).

We also found that the ratio of fluorescence intensity of labeled cells (FIL) by BOX/fluorescence intensity of unlabeled cells (FIU) ($r = 30$) was higher compared to cells stained by FDA ($r = 6.67$, Fig. 5). This difference allowed a much better separation of BOX-stained cells from wine debris compared to FDA-stained cells (Fig. 5).

Although both staining methods allowed bacteria undergoing MLF to be efficiently counted in white wine, with a detection limit of less than 10^4 CFU ml^{-1}, it was necessary to validate our protocol for bacteria in red wine.

Quantification of *O. oeni* in red wine

We inoculated filtered red wine with *O. oeni* (10^6 CFU ml^{-1}) stained with either FDA or BOX/PI and analyzed the suspensions by FCM and fluorescence microscopy (FM). FCM showed that the BOX-labeled cells were substantially more highly fluorescent than FDA-labeled cells (Fig. 6). The FCM analysis also showed that the ratio (FIL/FIU) of the BOX-labeled cells ($r = 25$) was higher than that of FDA-labeled cells ($r = 8.54$, Fig. 7).

We next inoculated filtered red wine with *O. oeni* (10^6 CFU ml^{-1}) and then analyzed samples collected during MLF by FCM (BOX/PI, quadrant 4) and by standard plate counts. We found strong correlations between the numbers of BOX/PI-stained cells determined by FCM and the cell numbers determined by plate counts ($R^2 \geq 0.97$, Figs. 8, 9). Our results demonstrate that BOX/PI staining and FCM could be applied to real-time monitoring of LAB numbers in wine undergoing MLF, which is not possible with plate counts or other available techniques.

On the other hand, we found that the detection and enumeration of *O. oeni* labeled with FDA was not efficient enough in red wine. Indeed, a great proportion of stained

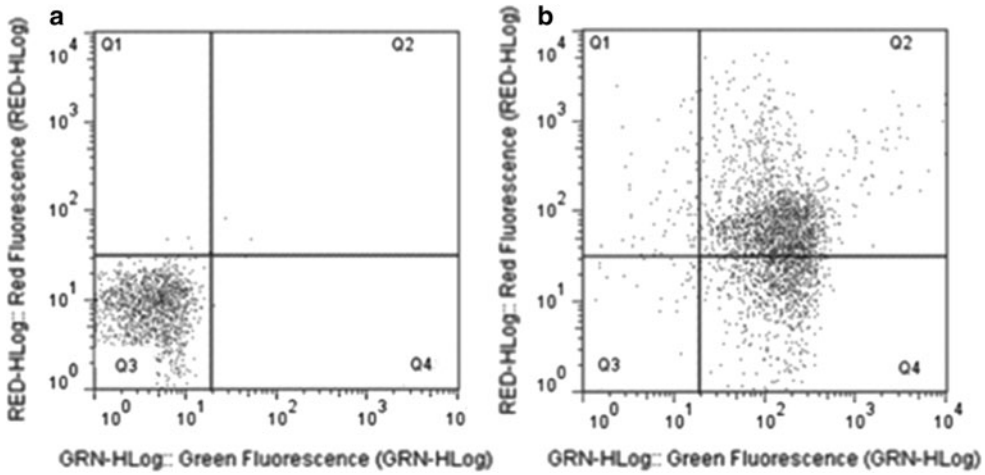

Fig. 3 FCM analysis of *O. oeni* cells grown in red wine for 9 days and stained with BOX/PI. Green fluorescence intensity is shown on the *x*-axes, and red fluorescence intensity is shown on the *y*-axes. *Dot plot* **a** shows the autofluorescence of unstained cells. *Dot plot* **b** shows the fluorescence of cells after staining with BOX/PI. PI-negative cells are contained in *quadrants 3* and *4*; PI-positive cells are contained in *quadrants 1* and *2*. BOX-positive cells are contained in *quadrants 2* and *4*; BOX-negative cells are contained in *quadrants 1* and *3*. PI fluorescence was measured at 630 nm (emission) and BOX at 525 nm (emission). Hardware compensation has been applied for adjusting the settings of the two-color analysis (BOX/PI)

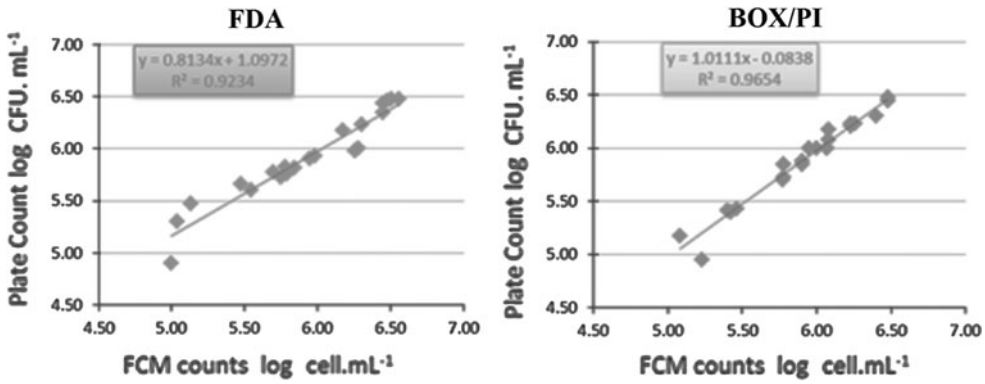

Fig. 4 The correlations between the numbers of *O. oeni* (starters I and II) in white wine determined by FCM and by standard plate counts are *linear*. The *straight lines* are the linear regressions calculated for the *plots* of the *O. oeni* counts in wine samples determined by FCM versus those determined by plate counts. The relationships produced high correlation coefficients (R^2) of 0.923 with FDA staining and 0.965 with BOX/PI staining (*quadrants 4*)

cells could not be differentiated from unstained cells (Fig. 7). We attempted to improve the FDA accessibility of the cells in red wine using different washing methods (PBS with and without Triton X-100) but found that it did not improve staining efficiency (data not shown).

Quantification of *O. oeni* in co-culture with *S. cerevisiae* during AF and MLF

MLF traditionally occurs shortly after the end of AF, but in current winemaking trends yeast and malolactic bacteria are co-inoculated at the start of AF. In such conditions, both yeast and bacteria could be stained by BOX/PI. For this reason, we assessed whether it was possible to discriminate these two microorganisms by FCM analysis using the scattering parameters.

Flow cytometry analysis of mixed cultures of malolactic bacteria and yeasts inoculated in red wine revealed that the size and the granularity of yeast is different from bacterial cells and wine debris, allowing yeast to be separated from bacteria cells and debris into two clearly distinguishable different zones based on light-scattering parameters (Fig. 10). These differences can be exploited to efficiently gate the specific cell types, allowing yeast cells and malolactic bacteria to be counted and separated from wine debris in a single analysis after they have been labeled with BOX/PI.

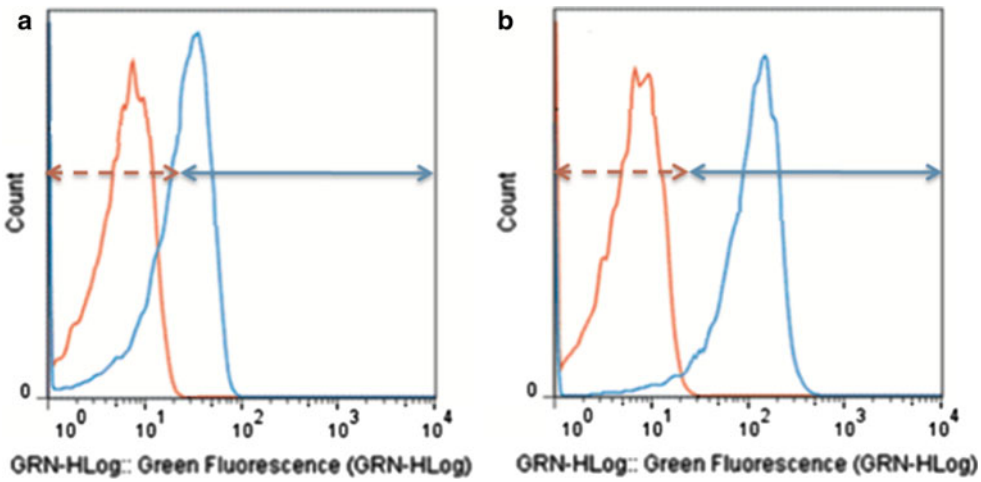

Fig. 5 FCM histograms of *O. oeni* cells grown in white wine and stained with FDA or BOX. Green fluorescence intensity (GRN-HLog) is represented on the *x*-axis, and cell counts are represented on the *y*-axis. *Panel* **a** shows the fluorescence of *O. oeni* before (*red arrow*; autofluorescence) and after (*blue arrow*) staining with FDA. *Panel* **b** shows the fluorescence of *O. oeni* before (*red arrow*; autofluorescence) and after (*blue arrow*) staining with BOX. *O. oeni* cells stained with BOX are more highly fluorescent than those stained with FDA (colour figure online)

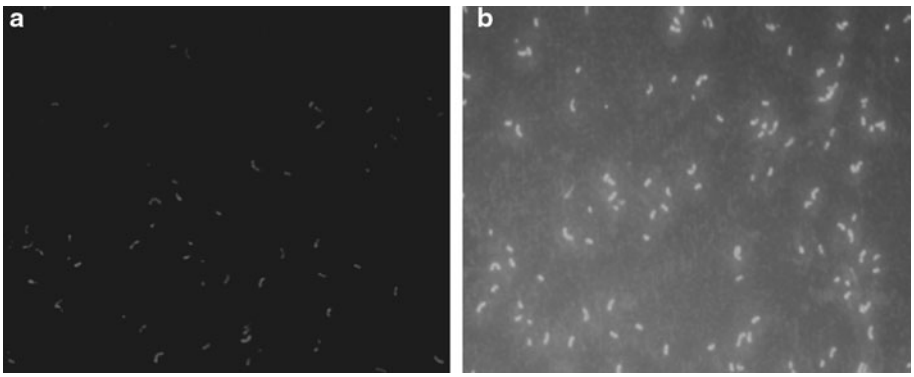

Fig. 6 Fluorescence microscopy of *O. oeni* cells stained with FDA (**a**) and BOX (**b**). *O. oeni* was cultured in red wine. Magnifications: ×100

We monitored two different co-cultures and compared the FCM results for *S. cerevisiae* and *O. oeni* to those obtained by standard plate counts. The cell numbers determined by FCM for both cell types correlated well with those obtained by plate count methods ($R^2 \geq 0.97$, Fig. 11).

After co-inoculation, the *S. cerevisiae* populations increased and reached their maxima ($>10^7$ cells ml^{-1}) after 2 days of growth. After day 7, the population decreased (Fig. 12).

During the growth phase of *S. cerevisiae*, the *O. oeni* populations decreased and stabilized rapidly during the first days of AF (between 5.10^5 and 10^6 CFU ml^{-1} for days 1–22). After the *S. cerevisiae* populations decreased (at sugar concentrations <1 g l^{-1}), the *O. oeni* populations increased from day 22 to 8.10^6 CFU ml^{-1}, and malic acid degradation signaled the beginning of MLF.

To assess whether *O. oeni* could be discriminated from other bacteria present in wine using FCM, we spiked wine containing *O. oeni* with either *P. damnosus* or *L. plantarum* and analyzed the light-scattering characteristics of the cell mixtures by FCM. The results showed that it was not possible to discriminate between the different types of bacteria by analyzing the light-scattering properties of the cells (data not shown).

Discussion

The ability to use a single instrument to rapidly conduct numerous microbiological assays offers obvious advantages for the wine industry over current methods in which culturing, microscopy or several dedicated instruments are needed. While cytometry-based methods exist to monitor yeast growth and viability during alcoholic fermentation in wine, there is no fast, efficient and affordable method that allows counting bacteria in white and red wines. Wine is a particular medium in that it contains numerous particles (positive signal by FCM using light-scattering parameters). Thus, care should be taken in FCM analyses because these

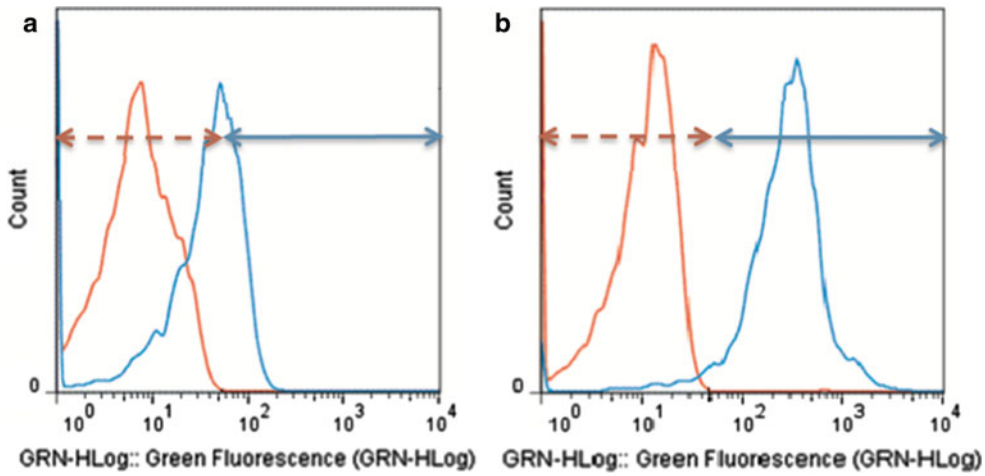

Fig. 7 FCM histograms of *O. oeni* cells grown in red wine and stained with FDA or BOX. Green fluorescence intensity (GRN-HLog) is represented on the *x*-axis, and cell counts are represented on the *y*-axis. *Panel* **a** shows the fluorescence of *O. oeni* before (*red arrow*; autofluorescence) and after (*blue arrow*) staining with FDA. *Panel* **b** shows the fluorescence of *O. oeni* before (*red arrow*; autofluorescence) and after (*blue arrow*) staining with BOX. *O. oeni* cells stained with BOX are more highly fluorescent than those stained with FDA (colour figure online)

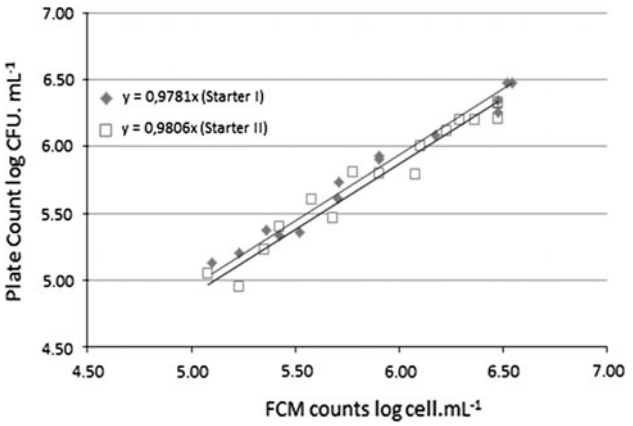

Fig. 8 The correlation between the numbers of *O. oeni* (starters I and II) in red wine determined by FCM and by standard plate counts is *linear*. The *straight line* is the linear regression calculated for the *plots* of the *O. oeni* counts in red wine samples determined by FCM (BOX/PI, *quadrant 4*) versus those determined by plate counts. The relationship produced a high correlation coefficient (R^2) > 0.97

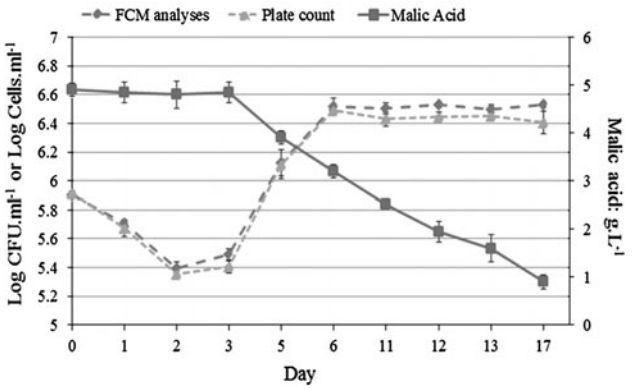

Fig. 9 Malic acid degradation versus growth of *O. oeni* (starter I) in red wine analyzed by FCM (BOX/PI, *quadrant 4*) and by plate count method. *Error bars* indicate the SDs of three independent experiments

particles are similar in size and density to bacteria and can produce misleading signals, especially in unfiltered wine. However, in this study, experiments were conducted in sterilized wine, i.e., free of particles, inoculated with yeast and bacteria. While bacteria could be separated from yeast in filtered wine because of the difference in their size and granularity; in the case of unfiltered wine, we found that it was not possible to discriminate between bacteria and wine debris by FCM using light-scattering parameters alone. For this reason, in order to count bacteria, cells need to be stained.

Malacrinò [22] previously showed that among various fluorescent dyes—rhodamine, calcein acetoxymethyl ester, 2′7′-bis(carboxyethyl)-5(6)-carboxyfluorescein

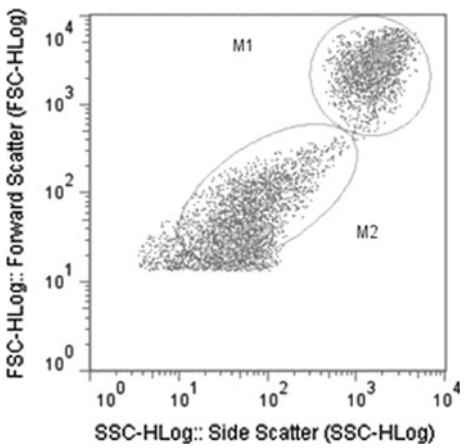

Fig. 10 Forward-scatter (FSC-H) and side-scatter (SSC-H) analysis of *S. cerevisiae* PB 2585 (M1) and *O. oeni* (starter I) (M2) in a ratio of 1:1

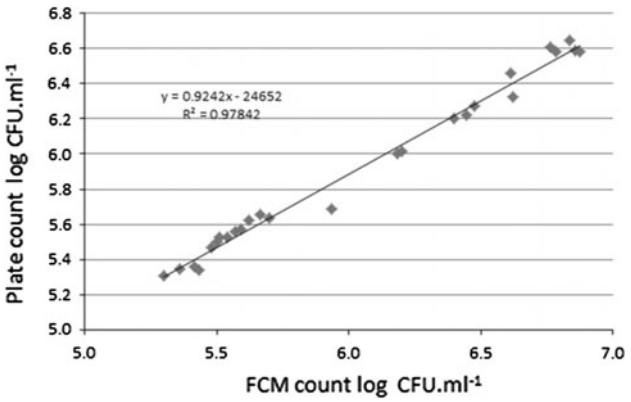

Fig. 11 The correlation between the numbers of *O. oeni* (starters I) determined by FCM and by standard plate counts is *linear*. The *straight line* is the linear regression calculated for the *plots* of the *O. oeni* counts present in *S. cerevisiae* determined by FCM (BOX/PI staining, *quadrant 4*) versus those determined by plate counts. The relationship produced a high correlation coefficient (R^2) > 0.97

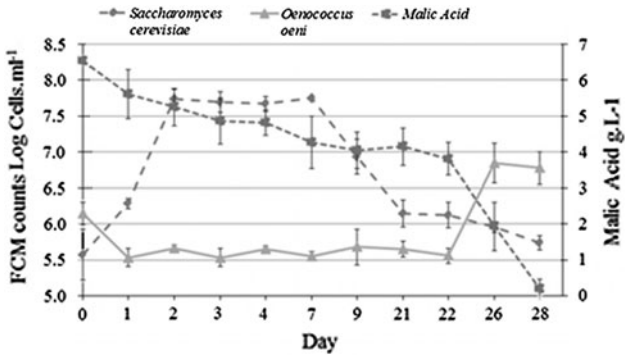

Fig. 12 Malic acid consumption versus growth of *O. oeni* (starter I) and *S. cerevisiae*. Cell counts were determined by FCM analysis. *Error bars* indicate the SDs of three independent experiments

acetoxymethyl ester and fluorescein diacetate—the highest fluorescence intensity was obtained with FDA. However, FDA did not stain all strains of *O. oeni*, which indicated that the use of this dye alone was unsuitable for enumerating bacteria in wine [22]. In addition, we demonstrate here that phenolic compounds can hamper bacteria labeling in red wines, as has been shown for other wine microorganisms [39]. Despite the use of different washing methods (PBS with and without Triton X-100), we were not able to efficiently stain bacteria with FDA in red wines. For these reasons, we investigated BOX/PI labeling, which has proven useful for detecting bacteria in different physiological states [40]. BOX is a anionic stain that binds to the CM only if it is depolarized. The good correlation coefficient between the plate counting and the FCM (quandrant 4) shows that cells are in a depolarized condition, which is due to the presence of some wine components such as ethanol, acidic pH (3.5), sulfites and phenolic compounds that affect the physical and chemical properties of the cytoplasmic membrane of LAB and which affect the membrane potential [17, 26–28, 36, 38].

We demonstrate that this staining has several advantages over FDA: (1) a higher fluorescence intensity that allows clear separation of stained bacteria from debris even in red wines; (2) this staining allows the labeling of all tested bacteria; (3) the double staining provides information on the physiological state of bacteria in wine.

Furthermore, our protocol allows labeling of yeast. Indeed, yeasts are easily stained with BOX/PI [41]. The growth kinetics of these yeast and bacteria during co-culture agrees with a previous report in which cell numbers were determined by plate counts [42]. This is very interesting in wine, since yeast and bacteria are naturally present together in wine during either alcoholic fermentation or malolactic fermentation [43]. Moreover, inoculating grape musts with wine yeast and lactic acid bacteria (LAB) concurrently in order to induce simultaneous alcoholic fermentation (AF) and malolactic fermentation (MLF) is a common trend nowadays [44]. Thus, our method allows monitoring both yeast and and bacteria in real-time during alcoholic and malolactic fermentation in white and red wine. This is a real advantage; if MLF is found to be sluggish or stuck, re-inoculation may be needed. Delaying re-inoculation by a week, especially at low SO_2 levels, is detrimental to wine quality. With FCM, the number of viable *O. oeni* cells in wine can be determined in 20 min, even in the presence of $>10^8$ yeast cells ml^{-1}. This rapid procedure is therefore a suitable method for monitoring *O. oeni* populations during wine-making and offers a detection limit of $<10^4$ CFU ml^{-1}. In contrast to classical microbiology techniques and molecular biology methods, which present constraints in terms of time and cost, FCM allows the timely application of corrective measures to regulate bacterial growth and improve the control of MLF in wine.

Must or wine may also contain acetic acid bacteria or other LAB such as *Pediococcus damnosus* or some *Lactobacillus plantarum* [45]. However, our methods do not address the presence of other microorganisms that are found in wine, such as the spoilage bacteria *P. damnasus* and some *L. plantarum*. Despite this disadvantage, our staining and FCM methods allow *O. oeni* to be followed in musts or in wine and can be used to determine the viability of *O. oeni* and *Saccharomyces cerevisiae* populations during AF and MLF in cases of inoculated or natural fermentations. Unfortunately, in cases of contamination, such as those encountered in case of stuck fermentation, these methods cannot be used to distinguish between *O. oeni* and the other types of spoilage bacteria. However, this is not a serious issue during fermentation because normally these bacteria only cause spoilage of wine during aging in the cellar and after bottling [1].

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