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Use of Flow Cytometry To Follow the Physiological States of Microorganisms in Cider Fermentation Processes

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The flow cytometry (FC) technique used with certain fluorescent dyes (ChemChrome V6 [CV6], DRAQ5, and PI) has proven useful to label and to detect different physiological states of yeast and malolactic bacterium starters conducting cider fermentation over time (by performing sequential inoculation of microorganisms). First, the technique was tested with pure cultures of both types of microorganisms grown in synthetic media under different induced stress conditions. Metabolically active cells detected by FC and by the standard plate-counting method for both types of microorganisms in fresh overnight pure cultures gave good correlations between the two techniques in samples taken at this stage. Otherwise, combining the results obtained by FC and plating during alcoholic and malolactic fermentation over time in the cider-making process, different subpopulations were detected, showing significant differences between the methods. A small number of studies have applied the FC technique to analyze fermentation processes and mixed cultures over time. The results were used to postulate equations explaining the different physiological states in cell populations taken from fresh, pure overnight cultures under nonstress conditions or cells subjected to stress conditions over time, either under a pure-culture fermentation process (in this work, corresponding to alcoholic fermentation) or under mixed-fermentation conditions (for the malolactic-fermentation phase), that could be useful to improve the control of the processes.

Analysis of microbiological samples from fermentation processes in the beverage industry (beer, wine, and cider) by traditional indirect, culture-based standard methods is time-consuming, and the methods do not produce direct information about the physiological state of the microorganisms. Moreover, the plate-counting method detects only cells able to form colonies under the conditions of the medium that is used, ignoring the presence of cells that do not form colonies but are nevertheless metabolically active (6). As is known, standard laboratory culture media rarely resemble natural environmental conditions (32). The application of flow cytometry (FC) with fluorescent dyes is faster and more direct and has made it possible to distinguish stages beyond the classical definition of viability generally demonstrated by culturing (24). FC can be used to quantitatively measure the optical characteristics of cells as they pass, in single file, into a focused beam of light (for a review, see reference 33). As particles pass through the beam, three parameters are measured: forward light scatter, side angle light scatter, and fluorescence at selected wavelengths. Light scatter is related to cell mass, structure, surface properties, and the optical density of the internal medium. A variety of fluorescent dyes may be used to detect structural or functional cellular properties. In this way, different cellular functions, such as reproductive growth, metabolic activity, and membrane integrity, can be detected, allowing a definition of the term viable-but-not-culturable cells (VBNC) (24) (other authors have called them active but nonculturable). Previous studies have reported that there are two major adaptations that cells undergo during the formation of VBNC states: cell wall toughening and DNA condensation (29). The detection of metabolic activity provides presumptive evidence of reproductive growth by the demonstration of enzyme activity, such as esterases, along with membrane integrity. In the absence of metabolic activity, it is still possible to determine membrane integrity by either dye retention or dye exclusion, the latter using propidium iodide (PI). Cells without an intact membrane cannot maintain or generate the electrochemical gradient that generates the membrane potential and can be considered dead cells (24). The use of FC techniques was reported for the "at-line" study of *Escherichia coli* fermentations (14), which detected a considerable drop in cell viability (about 20%) during the latter stages of small-scale (5-liter), well-mixed fed-batch fermentations.

Flow-cytometric applications using different dyes have been compared to standard methods to assess yeast cultures in baking, wine making, cider making, and brewing (1, 5, 7, 8, 10, 11, 17, 19). The technique has also been used to study the membrane integrity of ethanol-stressed *Oenococcus oeni* cells (9). Most studies of FC applications have focused mainly on microorganims as pure cultures, and only a small number have analyzed mixed populations over time (30).

The purpose of this work was to apply the FC technique in combination with certain fluorescent dyes (ChemChrome V6 [CV6], DRAQ5, and PI), not only to compare it to standard culturing methods, but mainly to gain insight into the physiological state of the microorganisms involved in cider fermentation (in this work, a yeast pure-culture fermentation process followed by a mixed culture of yeast and malolactic bacteria) throughout the process. To this end, the technique was first tested with pure cultures of both types of microorganisms grown in synthetic media under different conditions (in fresh

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overnight cultures, in heat-killed cells, or in mixtures containing fresh and heat-killed cells).

MATERIALS AND METHODS

Microorganisms. A commercial active dried yeast strain, *Saccharomyces cerevisiae* var. *bayanus* (strain Pasteur Institute, Paris, 1969, "Champagne," supplied by Novo Ferment, Switzerland), was used. The malolactic bacteria were isolated in the cellar of the Asturian cider-making firm Sidra Escanciador, S.A. (Villaviciosa, Spain), and maintained lyophilized and frozen (in 20% [vol/vol] glycerol at −20°C). Based on physiological and biochemical characteristics, the strain was previously considered an *Oenococcus oeni* strain. Molecular methods were applied to test the identification of the strain. PCR amplification of 16S rRNA genes was performed using the eubacterial universal primer pair 63f and 1387r, as previously described (20), followed by DNA sequencing. The sequence obtained was identified by database comparison (a BLAST search at http://www.ncbi.nlm.nih.gov), revealing a sequence identical (100% identity in 763 nucleotides) to the *Lactobacillus hilgardii* 16S rRNA sequence (IOEB0204). Therefore, the application of molecular techniques showed the need to reclassify this strain as a member of the species *L. hilgardii* (13).

Culture conditions. First, microorganisms were grown as pure cultures in synthetic media. YPD (yeast extract, peptone, D-glucose) broth was used for yeast culture under aerobic conditions (250 rpm in a shaker; New Brunswick Scientific) at 28°C for 18 h. De Man-Rogosa-Sharpe broth (Biokar, France) was used for the malolactic bacteria, without agitation, at 30°C overnight. Cell suspensions (washed and diluted as described below) were seeded in triplicate in statistically significant dilutions on YPD or De Man-Rogosa-Sharpe plates and incubated at 30°C for 48 h and 4 days, respectively.

Throughout the laboratory fermentations, yeast and bacteria were followed in each flask by counting viable cells on petri plates. The plates were incubated for 2 or 7 days, respectively, before colony enumeration.

Staining for flow cytometry analysis. Cells were collected by centrifugation (13,000 × *g*; 5 min), washed twice in phosphate-buffered saline (PBS) (pH 7.4, filtered at 0.22 μm), and adjusted to densities corresponding approximately to 10^5 to 10^6 cells ml^{-1} in the same buffer. Then, 200 μl of the same cell suspensions was added, separately, to the different staining solutions used, which were freshly prepared and filtered at 0.22 μm, mixed, and incubated in the dark. Total counts of cells were determined by staining with DRAQ5 (Biostatus Ltd., United Kingdom), a red-fluorescent cell-permeable DNA probe (31), to differentiate the microorganisms from other particles in samples (background). The stock (5 mM) was diluted 3:1,000 with sterile distilled water, 65 μl was added to the cell suspension, and the mixture was incubated for 10 min in the dark. CV6 (Chemunex, France), a fluorogenic ester converted to free fluorescein by esterase activity, was used to stain viable cells (25, 26). The stock (concentration not given by the manufacturer) was diluted 1:10 in sterile (0.22-μm-filtered) distilled water, 2 μl was added to the cell suspension, and the mixture was incubated for 10 min in the dark. PI, a fluorescent nucleic acid dye, was used to stain cells with compromised membrane integrity (9, 18). The stock (1-mg ml^{-1} solution in water; Molecular Probes) was diluted in sterile distilled water and added to the cell suspension at 10 μg ml^{-1} (final concentration) or 5 μg ml^{-1}, and the mixture was incubated for 30 min in the dark. Samples were immediately analyzed in the cytometer. For use as controls, fresh overnight cultures at exponential growth phase, heat-killed cells (the same fresh cultures but heated for 3 min at 65°C for bacteria or for 5 to 10 min at 80°C for yeast and immediately cooled on ice), and mixtures containing fresh and heat-killed cells (1:1) were analyzed by FC. Sample sonication was tested under different conditions. Samples were held in the "hot spot" (23, 28) of a sonication bath for different time intervals before analysis. Assays were carried out in parallel for the same bacterial samples subjected to four different treatments: two PBS washes, two PBS washes plus sonication for 15 s, two PBS washes plus sonication for 30 s, and finally, four PBS washes. These treatments were tested for cultures at both exponential and stationary phases in duplicate independent experiments.

Flow cytometry. Experiments were carried out using a Cytomics FC 500 (Beckman Coulter) with 488-nm and 633-nm excitation from an argon ion laser. Parameters were expressed on a logarithmic scale. Fluorescence of cells stained with DRAQ5 was recovered in the FL4 channel (675 nm), whereas those for CV6 and PI were collected in FL1 and FL3 (525 nm and 610 nm), respectively. A compensation setting was not applied. The detection threshold was set at the medium rate. Fluorescent microspheres (Perfect Count; Cytognos, Spain) were used as internal standards in each sample, following the supplier's recommendations for ratiometric counting (24). In order to obtain a significant number of cells to ensure the efficiency of the test, 2,000 microspheres were acquired in each analysis. Analyses were performed in triplicate for each dye, including an unstained sample as a control. Data analysis was carried out using the program Cytomics RXP Analysis (Beckman Coulter). If less than 100 positively stained cells (with PI or CV6) were detected, the result was not considered, due to the statistical counting error (24).

Microscopy. Samples were observed using a confocal laser microscope (ultraspectral Leica TCS-SP2-AOBS) or a fluorescence microscope (Leica DMR-XA) coupled to an image processor (Leica Q550) to confirm staining of the different types of cells used.

Fermentation conditions. Concentrated (bright, enzymatically treated) apple juice (70.8°Brix) supplied by Covillasa S.A. (La Almunia Doña Godina, Spain), was reconstituted with distilled water (1:6) with a specific gravity of approximately 1,064 g $liter^{-1}$ (pH 3.65). Juice sterilization was performed by a tangential-flow filtration device (Pellicon; Millipore) using a 0.22-μm polyvinylidene difluoride Durapore membrane (Pellicon 2 membrane cassette filter; Millipore) connected to a peristaltic pump.

Fermentations were carried out in duplicate without agitation in presterilized 1-liter cylindrical flasks filled to capacity.

Yeast active dried preparation was rehydrated in sterile apple juice at room temperature and grown under aerobic conditions at 250 rpm in a shaker (New Brunswick Scientific) at 28°C for 18 h. To start alcoholic fermentation, the apple must was inoculated with yeast (10^6 CFU ml^{-1}). The temperature was maintained at 16°C until completion of alcoholic fermentation (when the specific gravity reached approximately 1,005 g $liter^{-1}$).

To start malolactic fermentation (MLF) at the end of alcoholic fermentation, the temperature was fixed at 22°C and cells were added to the flasks (10^7 CFU ml^{-1}). For use as the inoculum, bacterial cells were collected from a pure culture in the same apple juice but supplemented with 0.5% (wt/vol) commercial yeast extract (Biokar, France) at the end of the exponential phase, centrifuged (3,000 × *g*; 10 min; Kubota 6700, Japan), washed in saline solution, and then suspended in the fermentation media.

Throughout both alcoholic and malolactic fermentation processes, samples were taken aseptically at approximately half the height of the flasks. It should be noted that, since the fermentations were carried out statically and sampling was performed without disturbing the fermentation media, only the biomass in suspension was considered. For biomass estimation by FC and plating methods, samples were washed and diluted as described above. Finally, the results were corrected by the dilution factor applied.

Chemical analysis. Fermentation samples were filtered immediately through a 0.45-μm membrane. The specific gravity was measured using a pycnometer. Until analysis was performed, the samples were frozen (−20°C) in 2-ml vial replicates. Malic and lactic acids were analyzed by high-performance liquid chromatography (Alliance 2690; Waters) with a photodiode array detector (Waters 996), as described previously (27).

RESULTS AND DISCUSSION

Test of the method (control samples). In a first set of experiments, control samples containing cells from pure yeast or malolactic cultures in synthetic media were used. Fresh overnight cultures at exponential growth phase, heat-killed cells, and mixtures containing fresh and heat-killed cells were analyzed (as CFU ml^{-1}) by FC and by culture on solid media. Our aim was, in the first place, to observe the staining patterns of each type of microorganism under different induced physiological conditions, such as the unstressed state (fresh overnight exponentially growing cells), stressed or dead (represented in this case by heat treatment), and mixtures containing both types. To confirm staining of the microorganisms, a confocal laser microscope was used in transmission and fluorescence modes. Overlaid images of the two modes obtained by the confocal laser microscope allowed checking of the expected staining patterns in the controls (results not shown).

In triplicate analyses, the FC technique gave accurate results (with a coefficient of variation near 1%) compared to plating (coefficient of variation, between 1% and 10% in statistically significant dilutions).

The results for active cells (stained with CV6) detected by flow cytometry and by the standard plate-counting assay for

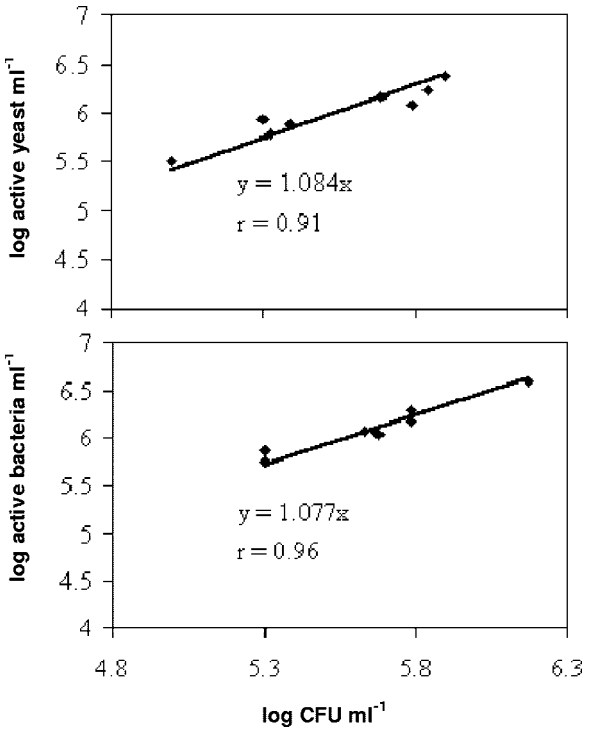


FIG. 1. Correlation between metabolically active (CV6-stained) yeast and malolactic bacteria obtained by FC and plate-counting methods, for pure cultures growing at exponential phase.

yeast and malolactic bacteria in overnight cultures at exponential phase in synthetic media are represented in Fig. 1. Good correlations between the techniques were achieved for cultures at this stage, although slightly higher values were reached for active cells detected by FC using CV6, as reported for the assessment of bacterial viability in water (25). The differences found may be attributed to the number of cells analyzed by each technique and, presumably, the presence of residual esterase activity. At this stage, the number of cells stained with CV6 corresponded well to the total number of cells stained with DRAQ5. CV6 can be considered a suitable dye for staining both *S. cerevisiae* and lactic acid bacteria (Fig. 2a and b). It was previously reported (19) that the fluorescence intensities of malolactic bacteria varied considerably, depending on the dye and the strain; some strains could not be labeled at all. In that work, fluorescein diacetate (FDA) gave the best results among the dyes tested. The same authors reported that FDA gave the best results for staining yeasts belonging to the species *S. cerevisiae* and *Saccharomyces bayanus*. However, it has been reported that FDA is not an ideal fluorescent substrate, as bacteria retain fluorescence poorly, resulting in weaker staining and increased background (33). da Silveira et al. (9) reported the use of the derivative 5(6)-carboxyfluorescein diacetate (cF) for staining *O. oeni* cells, with improved retention ability (33), although it should be noted that the cleavage product of this dye can be actively extruded from *S. cerevisiae* (4, 10).

A subpopulation of yeast stained by CV6 showing lower fluorescence intensity and unable to grow on plates was detected in the heat-killed controls, as shown in mixtures containing fresh and heat-killed yeast (Fig. 2c), which may be related to residual esterase activity (24). In the bacterial heat-killed controls, without colony-forming ability, no cells were labeled by CV6 staining.

In previous experiments, Sytox Orange (Molecular Probes), a high-affinity nucleic acid stain that penetrates cells with compromised membranes and that will not cross the membranes of live cells, was tested as an indicator of membrane integrity. However, the dye gave inconsistent results in FC analysis, especially for staining the malolactic strains (results not shown). When labeled malolactic cells were observed under the fluorescence microscope, a photobleaching effect was seen (results not shown). TOTO-1, a membrane-impermeant nucleic acid stain that labeled membrane-permeabilized cells, was also tested, as reported previously (6), but the dye did not give the expected staining patterns for malolactic bacteria when the control samples were used. PI was selected.

When using PI to detect dead cells in heat-killed controls (for both yeast and bacteria), in which colonies were not detected by plating, only one population showing a high fluorescence signal was detected (Fig. 2d). The total number of PI-stained cells corresponded well to the total number stained with DRAQ5. In exponentially growing malolactic control bacteria, a second subpopulation showing lower fluorescence intensity was detected in PI-stained samples. In mixtures of fresh and heat-killed bacterial cells, these two subpopulations of PI-stained cells showing different intensities could be perfectly distinguished (Fig. 2e). Samples analyzed by FC were also observed with a fluorescence microscope. Bacterial cells corresponding to the heat-killed control (without growth on plates) showed only clear, bright, positive staining of well-defined cells (Fig. 3a). Diffuse fluorescent signals were found in the exponentially growing control (Fig. 3b), and as expected, both types were detected in the mixtures (Fig. 3c). These diffuse signals indicated that, as previously reported for *E. coli* (16) and *S. cerevisiae*, PI had not fully entered the cells, which proved to be predominantly culturable when the sorted cells were plated (10). In our work, using PI at concentrations of 10 and 5 $\mu g\ ml^{-1}$, the same effect was observed. Therefore, in this work, only the subpopulation showing higher fluorescence intensity was considered to be positive PI-stained bacterial cells.

Based on the results observed in the controls and in cider samples inoculated simultaneously with high numbers of both microorganisms (results not shown), the regions showing the different fluorescence signals were gated for the dyes employed and the microorganisms tested (based on their different light-scattering profiles).

Malolactic bacteria are able to form chains. In order to avoid problems associated with cell aggregation, sonication of samples was tested, as previously proposed (23, 28). Samples were held in the "hot spot" of a sonication bath before analysis for different time intervals. Taking into account the risk of physically damaging the cells, assays were carried out in parallel for the same bacterial samples submitted to four different treatments. These treatments were tested for cultures at both exponential and stationary growth phases in duplicate independent experiments and were analyzed by FC and plate-counting methods. The dead-cell/active-cell ratio (as a percentage) and, in the same terms, the viable-cell/active-cell ratio were considered and compared to the treatment with two PBS washes. An

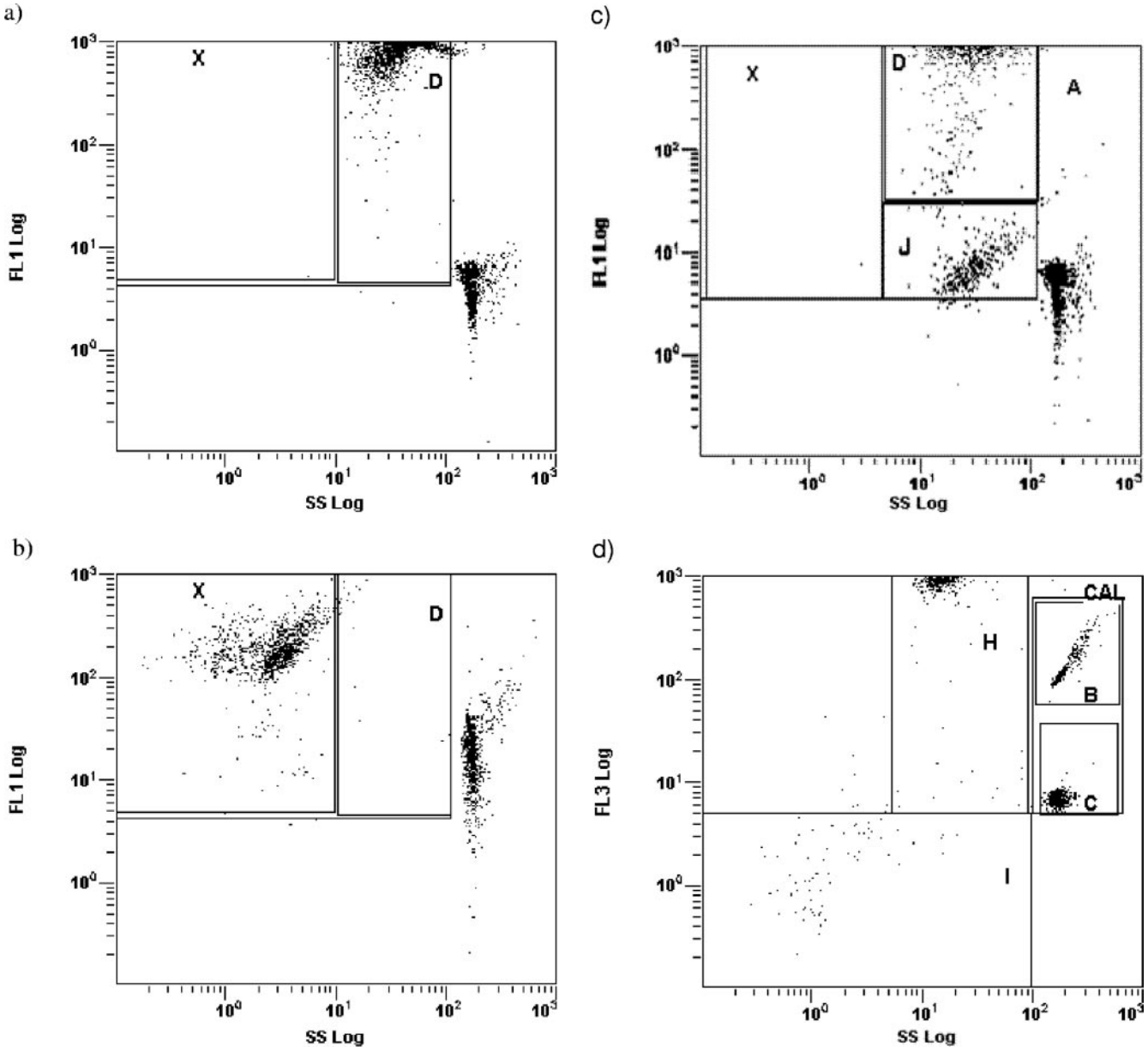


FIG. 2. Pure cultures. (a) *S. cerevisiae* at mid-exponential phase stained by CV6 (D). (b) CV6-stained malolactic bacterial cells at the same physiological stage (X). (c) Mixtures containing fresh and heat-killed yeast stained by CV6 (D and J). The lower subpopulation (J) was detected in heat-killed controls without colonies on plates. (d) PI-stained yeast in heat-killed controls (H). (e) Malolactic bacteria stained by PI showing two distinct subpopulations with different fluorescence intensities (E and I) (corresponding to the micrograph in Fig. 3c).

increase in the dead/active ratio was observed when sonication was applied to cultures at exponential phase, suggesting progressive damage and permeabilization of cells, although this effect was almost negligible for cultures at stationary phase. The pretreatment with four PBS washes occasionally led to losses in the total number of cells, probably due to excessive sample manipulation (results not shown). If disaggregation had taken place during the treatments, the counts of total and viable cells would have increased in relation to the two-PBS-wash samples. This effect could not be assessed (results not shown). Therefore, it was considered that the sonication pretreatments were not suitable for the malolactic bacteria. It should be noted that sample manipulations following dilution and two PBS washes, including vortexing, as performed in this work, may help to disrupt aggregated cells at the time of the cytometric analysis, as previously stated (7). Thus, the sonication pretreatment was not applied in subsequent experiments.

Cider fermentation process—description of physiological states. Changes in the specific gravity during alcoholic fermentation are shown in Fig. 4. During this phase, the evolution of total cell counts (cells stained by DRAQ5), metabolically active cells (CV6 labeling), and PI-stained yeast cells by FC was followed, along with viable yeast, by plating (Fig. 5). Yeast cells were basically settled by day 20 of the alcoholic fermentation.

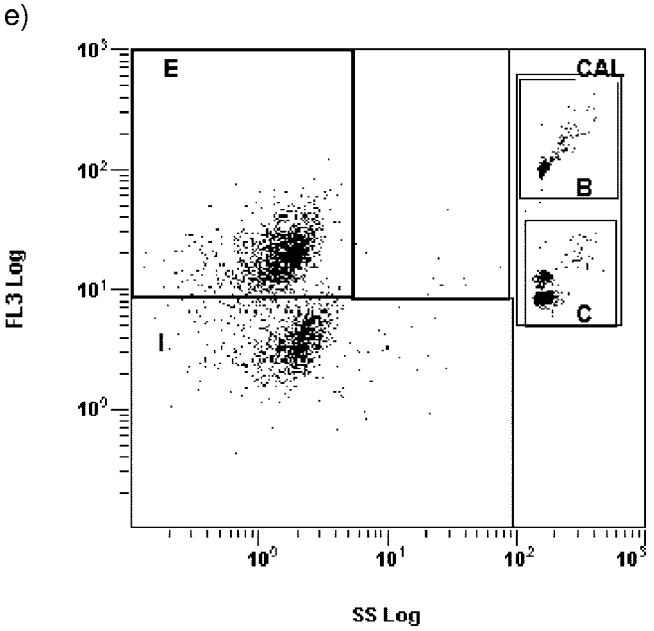


FIG. 2—*Continued.*

Under fermentation conditions, combining the results obtained by plating and FC, different subpopulations of yeast could be distinguished, differing from the observations made of the control samples corresponding to nonstressed, fresh overnight cultures in synthetic media. Percentages of yeast cells stained by CV6 and PI (CFU and VBNC) in relation to the total number of yeast cells (stained by DRAQ5) are presented in Table 1. Plate counting quantified the proportion of culturable yeast cells, which corresponded to a subpopulation of the metabolically active cells, in this case representing only 30 to 40% of the total yeast cells during the whole process (Table 1). Obviously, although the capability for reproductive growth is important during the first stage of alcoholic fermentation for the implantation of the starter culture, the main function of yeast is fermentation. CV6-stained yeast cells, as an indicator of metabolically active yeast, remained near 90% until day 12, dropping to 70% at the end. Detection of enzyme activity, as performed in this work, indicated that the cells had synthesized these enzymes in the past and showed the ability to maintain them in an active form (24). Enzymes present in the fermentation media may still be in an active form. The percentage of the VBNC subpopulation obtained (calculated as active yeast cells minus culturable yeast cells) was high, indicating that these cells may well contribute to the fermentation activity, as observed previously (6). During alcoholic fermentation, the addition of CV6- and PI-stained cells was approximately 100% of the total number of cells detected by DRAQ5. The PI-stained subpopulation remained near 10 to 15%, increasing

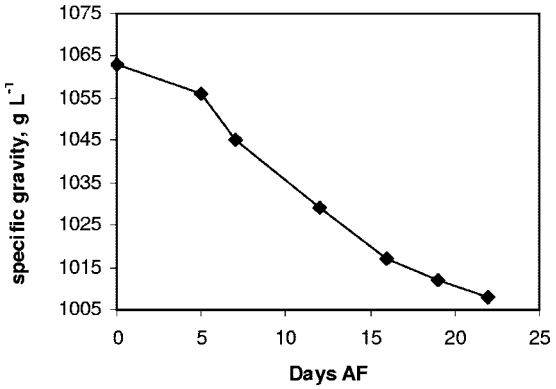


FIG. 4. Specific gravity during alcoholic fermentation (AF).

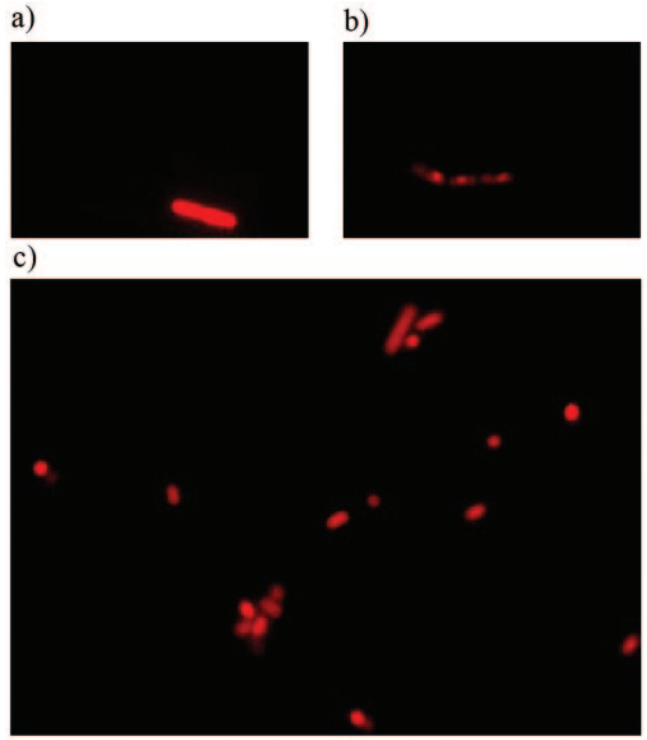


FIG. 3. Micrographs, obtained by fluorescence microscopy, of PI-stained malolactic bacteria showing different fluorescence patterns. (a) Heat-killed controls without colonies on plates, corresponding to fully PI-stained dead cells. (b) Diffuse fluorescence signals in exponentially growing controls in which PI had not fully entered the cells. (c) Mixtures containing fresh and heat-killed cells, showing both types.

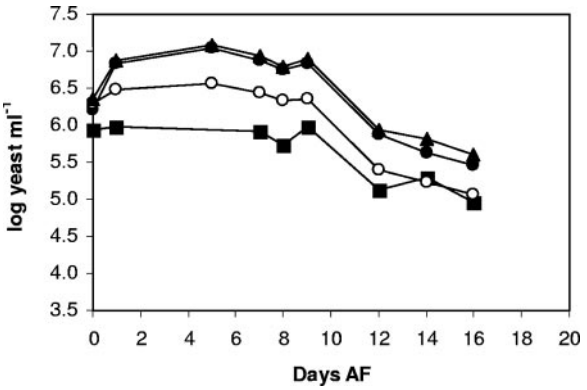


FIG. 5. Evolution of total counts (▲) (DRAQ5-labeled), metabolically active (●) (CV6-stained), and membrane-permeabilized (■) (PI-stained) yeast cells by FC, along with plating (○) (CFU) of yeast during alcoholic fermentation (AF).

TABLE 1. Results combining FC and plating methods for yeast during alcoholic fermentation

Day AF[a]	% CV6 stained	% PI stained	% CV6 + PI stained	% CFU	% VBNC
1	90	13	103	40	50
5	89	13	102	30	59
7	85	9	94	32	53
8	90	9	99	34	56
9	87	12	99	29	57
12	88	15	104	28	60
14	64	31	95	26	38
16	73	23	96	29	43

[a] AF, alcoholic fermentation.

after day 12, just when the active yeast decayed. This subpopulation represented the permeabilized yeast, which may well be considered dead cells in this context.

The malolactic bacterial starter culture was added following a sequential-inoculation model (i.e., once alcoholic fermentation was completed), as previously performed (13). The MLF developed successfully (Fig. 6). Malic acid could not be detected after day 7 postinoculation with the malolactic bacteria, while the lactic acid concentration increased in the fermentation media. In the same way as for alcoholic fermentation, during MLF, microorganisms were followed by both techniques (Fig. 7). Due to the model of inoculation and dilution performed, yeast cells could not be found in significant numbers in the samples. The percentages of malolactic bacteria stained by CV6 and PI (CFU and VBNC) in relation to the total counts of bacteria stained by DRAQ5 are shown in Table 2. Combining both techniques, in this fermentation process, up to four different subpopulations in the malolactic culture might be detected (Fig. 8c). The subpopulation capable of growing on plates represented 20 to 30% (lower than the yeast subpopulation during alcoholic fermentation) and dropped once malolactic fermentation was finished. The metabolically active subpopulation stained by CV6 remained very high during the process, surprisingly near 100% of the total DRAQ5-stained bacterial cells. The initial PI-stained subpopulation increased from 10% to 35% at the end of the malic acid bioconversion. The VBNC subpopulation increased during the process, reaching higher values than those observed in yeast for alcoholic fermentation. As is known for cider fermentation, low pH, the

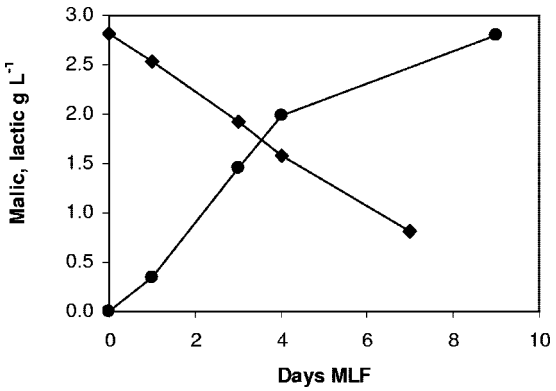


FIG. 6. Malic (◆) and lactic (●) acid evolution during MLF.

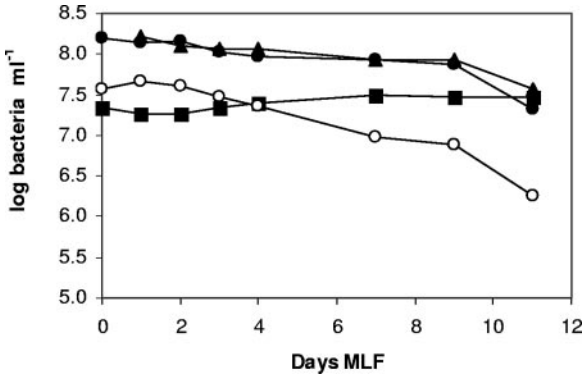


FIG. 7. Malolactic bacterial cells stained by DRAQ5 (▲), CV6 (●), and PI (■) (detected by FC), along with viable counts by plating (○) (CFU) during MLF.

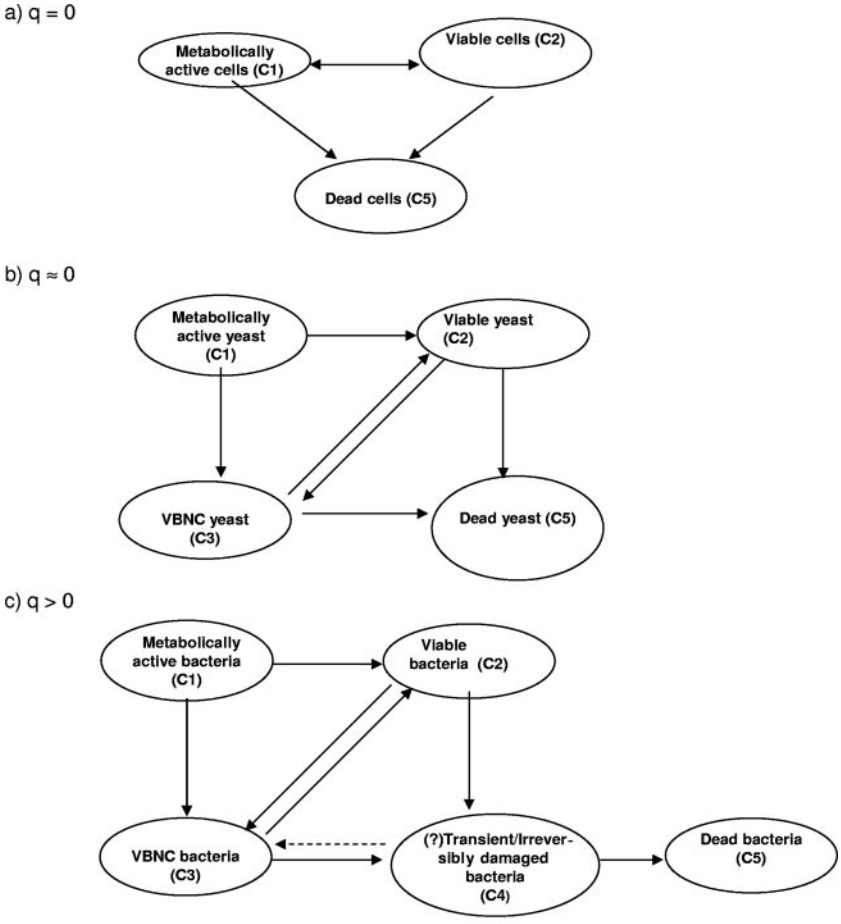
presence of ethanol and other inhibitors, nutrient deficiency, and interactions between yeast and bacteria influence the adaptation of the malolactic bacteria to the stress conditions in the fermenting must (13).

It should be noted that, during this phase, the additions of PI- and CV6-stained subpopulations exceeded the total number of DRAQ5-stained cells. Thus, a dually stained subpopulation might be considered, consisting of bacterial cells capable of being labeled by either CV6 or PI. Similar situations had been previously described with PI and ChemChrome Y in yeast (10) and PI-cF (as an indicator of enzyme activity and membrane integrity) double staining in bile salt-stressed bifidobacterial cells (3).

As has been mentioned, a potential error for FC analysis is the presence of clusters of cells. It is known that the presence of clusters is unlikely to affect the correlation between FC and plate-counting techniques, since both methods record clusters containing one or more viable cells as one viable unit (10). However, in simultaneous double-staining analysis, if one cell in a triplet is positive for a dead-cell marker, the whole aggregate will be registered as dead in spite of the two active cells. Since in this work single staining was performed, the presence of triplets could be registered as one positive event for CV6 and one for PI labeling, underestimating the triplet as one event labeled by DRAQ5. Therefore, the possibility that the calculated subpopulation of dually stained malolactic bacteria might be explained in such terms could not be completely dismissed. However, as indicated above, the assays carried out led us to consider that the standard two PBS washes performed

TABLE 2. Combination of results obtained by FC and plate counting for malolactic bacteria during MLF

Day MLF	% CV6 stained	% PI stained	% CV6 + PI stained	% CFU	% VBNC	% Double stained
1	85	11	96	28	57	
2	114	15	129	32	82	29
3	92	19	111	26	66	11
4	81	21	102	20	61	2
7	100	36	136	11	89	36
9	88	35	123	9	79	23
11	58	82	140	5	53	40



Definitions:
DRAQ5 = x
CV6 = y
PI = z
(y + z) − x = q

Equations:

a) exponentially growing non-stress conditions: q = 0
y = C1 ≈ C2
z = C5
b) stress conditions during cider fermentation: alcoholic fermentation (AF): q ≈ 0
y = C2 + C3
z = C5
x = C2 +C3 + C5
c) stress conditions during cider fermentation: malolactic fermentation (MLF): q >0
y = C2 + C3
z = C4 (?) + C5
x = C2 + C3 + C4 (?) + C5

FIG. 8. Diagram representing plausible significant subpopulations. (a) Exponentially growing, nonstress conditions. (b and c) Stress conditions during cider fermentation. (b) Alcoholic fermentation. (c) Malolactic fermentation. C2, plate counting as CFU ml^{-1}. The arrow directions indicate possible reversible states. Equations to determine each subpopulation are also shown.

were enough to avoid aggregation of malolactic bacteria. Presumably, triplets did not actually occur. Interestingly, a double-stained PI-cF subpopulation of ethanol-stressed *O. oeni* cells has been described (9). In that work, approximately 35% of the PI-stained cells also accumulated cF (quite near to the 30% dually stained CV6-PI subpopulation detected in most samples during MLF in cider), indicating a progressive change in the physiological status of *O. oeni* and showing intermediate membrane permeability. Similarly, results obtained during MLF in cider have shown a progressive change in the physiological status of malolactic bacteria. In that work, it was also shown that when cells were grown in the presence of 8% (vol/vol) ethanol and were then challenged for 25 min with 16% (vol/vol) ethanol, the subpopulation stained with PI-cF represented 47% of the total population. The authors also observed a positive effect of ethanol adaptation in cells grown in the presence of ethanol, although the sizes of the subpopulations of cells in an intermediate state of membrane damage (double-stained cells) were similar for both adapted and nonadapted cells. The double-stained subpopulation of ethanol-stressed cells revealed population heterogeneity. Interestingly, it has been shown that permeabilization may lead to a higher acid production rate (6).

Heterogeneity under stress conditions has also been described in *S. cerevisiae* (2) by FC. The presence of subpopulations in a commercial yeast strain not corresponding to distinct subspecies but representing different physiological responses to changing abiotic conditions during different stages of a brewing process on an industrial scale has been reported (22). In that work, the FC technique allowed changes in structural and functional parameters of the yeast cells, differing in their DNA and neutral-lipid contents, to be quantified. There has been increasing interest in the application of the FC technique in the field of microbial fermentation (15), since it is already possible to detect heterogeneity among individual cells in a population not detected by traditional methods.

As mentioned above, only a small number of studies have applied the FC technique to analyzing interactions in mixed cultures over time (12, 21, 30). In such studies, concordances were found between FC methods and plate counts when pure cultures were measured, but discordances were observed in mixed cultures, since much larger populations were detectable using FC than in agar plates (30). In the first work (12), differences were attributed to the presence of dead but intact cells. (19) Significant differences between enumerations by plate counting and FC were reported in winery samples, attributed to difficulties in direct detection of bacteria in wine and in sample preparation. In dairy fermentation starters and in probiotic products, substantially higher numbers for FC total cell counts than CFU were reported (6). Notably, it has been established that when starter cultures are subjected to various forms of sublethal stress, some cells may be injured and fail to grow on a medium adequate for the growth of unstressed cells, but they are capable of growth when they are given a suitable environment (6). Evidence of recovery of growth ability by damaged or injured, stressed cells by repairing or replacing damaged molecules under suitable conditions has been reported (3, 29).

Based on the results obtained in this work, equations were postulated (Fig. 8) to explain the different conditions observed. Different physiological states in populations were detected (i) for samples taken from fresh overnight pure cultures exponentially growing under nonstress conditions or cells subjected to stress conditions during fermentation over time, (ii) for alcoholic fermentation (in this work, a pure-culture fermentation process), and (iii) for malolactic fermentation (under mixed-fermentation conditions). In the last case, inhibitory compounds resulting from the mixed-fermentation conditions were present. Under stages shown in cases i and ii, FC data obtained could be used to estimate the number of culturable cells (those showing growth ability on solid media as traditionally considered) directly or simply by solving the two-equation system without applying the plating method. In the third case, four unknowns are present in a three-equation system, and thus, plating would be needed to separate the growth-capable subpopulation from the metabolically active subpopulation.

Further work focused on the application of FC in industrial-scale cider fermentation is needed to show the advantage of this technique under real conditions.

Conclusions. The application of FC to cider fermentation could be useful to determine other physiological states not considered by the traditional plate-counting method, with the consequent possible improvement of alcoholic and malolactic fermentation. Most available FC data are limited to pure-culture conditions, while data regarding fermentation or mixed-culture processes over time are still scarce. The role played by the VBNC microorganisms during cider fermentation should be further determined. Sampling is still generally performed by direct plating of the fermentation medium, with the aim of characterizing microorganisms taking part in complex fermentations. Especially in an enological context, this commonly used procedure can lead to erroneous estimates of the percentage of a given genus or strain in the process or simply to dismissing their contribution, even as an alternative microbiota. Accurate measurement techniques for active biomass estimation under real-time conditions is also essential for the control of the process, and related mathematical models are applicable to predict performance on an industrial scale. In developing other applications in future work, FC results must be related to ethanol and malic acid transformation efficiencies and even to the formation of other secondary products that influence taste. The industrial application of FC for control of the process under real fermentation conditions would require the use of simpler FC equipment.

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