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Monitoring of *Saccharomyces cerevisiae*, *Hanseniaspora uvarum*, and *Starmerella bacillaris* (synonym *Candida zemplinina*) populations during alcoholic fermentation by fluorescence *in situ* hybridization

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ABSTRACT

Various molecular approaches have been applied as culture-independent techniques to monitor wine fermentations over the last decade. Among them, those based on RNA detection have been widely used for yeast cell detection, assuming that RNA only exists in live cells. Fluorescence *in situ* hybridization (FISH) targeting intracellular rRNA is considered a promising technique for the investigation of wine ecology. For the present study, we applied the FISH technique in combination with epifluorescence microscopy and flow cytometry to directly quantify populations of *Saccharomyces cerevisiae*, *Hanseniaspora uvarum*, and *Starmerella bacillaris* during alcoholic fermentations. A new specific probe that hybridizes with eight species of *Hanseniaspora* genus and a second probe specific for *Starm. bacillaris* were designed, and the conditions for their application to pure cultures, mixed cultures, and wine samples were optimized. Single and mixed fermentations were performed with natural, concentrated must at two different temperatures, 15 °C and 25 °C. The population dynamics revealed that the *Sacch. cerevisiae* population increased to 10^7–10^8 cells/ml during all fermentations, whereas *H. uvarum* and *Starm. bacillaris* tended to increase in single fermentations but remained at levels similar to their inoculations at 10^6 cells/ml in mixed fermentations. Temperature mainly affected the fermentation duration (slower at the lower temperature) but did not affect the population sizes of the different species. The use of these probes in natural wine fermentations has been validated.

1. Introduction

Wine fermentation is an ecologically complex process, and it is widely accepted that the yeast population changes as the fermentation proceeds (Fleet, 2008; Wang and Liu, 2013). Yeast species play important roles in the wine-making processes: transforming sugar to ethanol, producing specific secondary metabolites, and finally, contributing to wine flavor characteristics. The main yeast involved in this transformation process belongs to the *Saccharomyces* (*Sacch.*) genus, but other wine yeasts can also be isolated during the process, producing varying impacts on the wine composition (Andorrà et al., 2012; Fleet, 2003). Most of the knowledge generated concerning wine yeast ecology has been derived from plating, which has incorporated molecular analysis in the past 25 years (Ribéreau-Gayon et al., 2006). However, in the past 15 years, culture-independent techniques have provided a new view of microbial ecology during the wine production process.

The culture-independent molecular techniques employed to study the ecology of wine yeasts have mainly been based on the detection of DNA in wine samples (Andorrà et al., 2010a; Cocolin et al., 2000; Hierro et al., 2006). However, it is possible to detect DNA from dead cells when using these techniques (Andorrà et al., 2010b; Hierro et al., 2006). Furthermore, the presence of viable but non-culturable (VBNC) or injured cells in wine (Divol and Lonvaud-Funel, 2005; Millet and Lonvaud-Funel, 2000) introduces a new bias into the analysis of the wine yeast ecosystem. More recently, a small modification of the same culture-independent methods, QPCR coupled with ethidium monoazide (EMA)/PMA (Andorrà et al., 2010b; Rawsthorne and Phister, 2009), has been proposed to reveal the actual viable yeast population structure and resolve the differences observed between plate counting and molecular results. Other methodologies based on RNA detection, such as RT-PCR-DGGE (Mills et al., 2002) and RT-QPCR (Hierro et al., 2006), have also been proposed. A main hurdle of these techniques is that all of these methods rely on a high-quality DNA/RNA extraction and PCR/QPCR operation to provide accurate results. Fluorescence *in situ* hybridization (FISH) probes directly hybridize with intracellular ribosomal RNA at specific sites, and the target cells with fluorescent signals can be easily observed and recorded by epifluorescence microscopy and flow cytometry (Amann and Fuchs, 2008). In addition to its simplicity and rapidity, the ability to observe the cell morphology by a microscope and the high sensitivity obtained using a flow cytometer (ten fluorescent cells can be detected among ten million non-fluorescent cells, as the flow cytometer

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can detect up to ten million events) make FISH a very promising technique for wine ecology studies (Andorrà et al., 2011; Branco et al., 2012; Díaz et al., 2010; Röder et al., 2007).

To our knowledge, only a few wine ecology studies have reported on the detection of yeast populations by FISH, mainly because of the difficulty of designing probes. Although early studies (Inácio et al., 2003; Stender et al., 2001; Xufre et al., 2006) were based on culture-dependent techniques, probes for several wine-related yeast species have been proposed, and the data can be used to design new probes for other species as well. Xufre et al. (2006) developed nine different probes for the detection of *Saccharomyces* and non-*Saccharomyces* wine yeasts. The first approximation of population monitoring during wine fermentation accomplished using FISH combined with flow cytometry was proposed by Andorrà et al. (2011). These authors found that the *Hanseniaspora* (*H.*) *guilliermondii* probe demonstrated a low fluorescent intensity compared to the *Saccharomyces cerevisiae* probe, whereas *H. guilliermondii* cells could be differentiated easily from *Sacch. cerevisiae* cells by flow cytometry based on the cell size and granularity. Upon analyzing the changes in the fluorescence intensity of the stained cells during the fermentation, the intensity of the *Sacch. cerevisiae* probes decreased while *H. guilliermondii* maintained a similar intensity as that of the initial conditions (Andorrà et al., 2011). Because of the high background fluorescence in wine, only high-intensity signal probes, which have only been applied for detecting *Dekkera bruxellensis* and *Sacch. cerevisiae* in direct wine sample analysis, can be used (Andorrà et al., 2011; Röder et al., 2007). Therefore, the development of specific probes with sufficient fluorescence is still required for further assays of yeast populations directly from wine samples by FISH.

Yeast diversity studies of red and white musts from our faculty cellar (Tarragona, Spain) have been performed for several years, with *Saccharomyces*, *Hanseniaspora*, and *Candida* (*C.*) comprising the main yeast genera detected. Thus, the present study aims to analyze the applicability of culture-independent FISH techniques coupled with epifluorescence microscopy and flow cytometry in determining changes in these yeast populations during alcoholic fermentations. Sterile musts were inoculated with *Sacch. cerevisiae*, *Hanseniaspora uvarum*, and *Starmerella* (*Starm.*) *bacillaris* (synonym *Candida zemplinina*) to perform single and mixed fermentations. In this study, we have detailed the design of new probes with improved fluorescence intensity for monitoring *Hanseniaspora* and *Starm. bacillaris* in wine fermentations. The fermentations were performed at two temperatures that are relevant from the wine-making point of view.

2. Materials and methods

2.1. Yeast strains

The yeast strains and species used in this study are listed in Table 1. Yeasts were grown overnight in YPD (1% yeast extract, 2% peptone, and 2% glucose, w/v) medium at 28 °C before use. All yeasts were identified by 5.8S-ITS-RFLP analysis according to Csoma and Sipiczki (2008) and Esteve-Zarzoso et al. (1999) and by sequence analysis of the D1/D2 domain of the 26S rDNA (Kurtzman and Robnett, 1998).

2.2. Design of oligonucleotide probe

Prior to designing the new probe, the published probes of *Sacch. cerevisiae*, *H. guilliermondii*, and *Candida stellata* (Xufre et al., 2006) were synthesized and checked by hybridization with target species. Because of the undesirable results of the probes for *H. guilliermondii* and *C. stellata* in our initial assays, new oligonucleotide probes for *Hanseniaspora* and *Starm. bacillaris* that target within the D1/D2 domain of the 26S rRNA were designed (sequences were obtained from the GenBank database, accession numbers are shown in Table 2). Species-specific sequences were selected according to the alignment by Clustal Omega (EMBL-EBI) and the accessibility map of *Sacch. cerevisiae* (Inácio et al., 2003). Subsequently, general and mismatch analyses with mathFISH (Yilmaz et al., 2011) were used to evaluate the sequences of these probes. To overcome a potential unspecific hybridization site, a competitor was designed to combine with the mismatched

Table 1
Yeast strains and species used in this study and the specificity results for the three probes in pure cultures (sources: CECT, Spanish Type Culture Collection, Universitat de València; CBS, Centraalbureau voor Schimmelcultures, Delft, Netherlands; MCYC, Microbiology Collection of Yeast Cultures, Universidad Politécnica de Madrid; NS, natural isolates from our group collection; NCYC, National Collection of Yeast Cultures, Norwich, United Kingdom). Positive (+) or negative (−) hybridization is indicated.

Strains	CECT designation	Other designation	Isolation source	Specificity results for each probe		
				Sce-FITC	H8b-FITC + H8b-Com	Sba-FITC
Candida boidinii	11168	CBS 6990	Tepache	−	−	−
	1014[T]	CBS 2428	Tanning fluid	−	−	−
Candida mesenterica	1025	CBS 602	Brewery	−	−	−
Candida sake	10034	MCYC 123	Feces of sheep	−	−	−
	1044	CBS 617	Lambic beer	−	−	−
Candida stellata	11918[T]	CBS 157	Wine grapes	−	−	−
Starmerella bacillaris	11046	CBS 2649	Grape juice	−	−	+
(synonym *Candida zemplinina*)[a]	11109	CBS 1713	Wine	−	−	+
	–	NS c	Grape must	−	−	+
	–	NS d	Grape must	−	−	+
Hanseniaspora opuntiae	11027[b]	NCYC 2380	Grape must	−	+	−
Hanseniaspora guilliermondii	11029	CBS 465	Infected nail	−	+	−
	11102	CBS 1972	Grape juice	−	+	−
Hanseniaspora osmophila	11206	CBS 313	Ripe Riesling grape	−	−	−
Hanseniaspora uvarum	1444[T]	CBS 314	Muscadet grape	−	+	−
	10389	MCYC 1857	Grape juice	−	+	−
	11105	CBS 2589	Grape must	−	+	−
	11106	CBS 5073	Wine grape	−	+	−
	11107	CBS 8130	Grapes	−	+	−
	–	NS b	Grape must	−	+	−
Hanseniaspora vineae	1471	CBS 6555	Grape juice	−	−	−
Metschnikowia pulcherrima	–	NS f	Grape must	−	−	−
Saccharomyces cerevisiae	1942[T]	CBS 1171	Beer	+	−	−
	–	NS a	Grape must	+	−	−
Torulaspora delbrueckii	–	NS e	Grape must	−	−	−

[T] means Type strain.
[a] A new name change according to Duarte et al. (2012); the two strains CECT 11046 and CECT 11109 were preserved as *C. stellata* until November 2012 by CECT.
[b] CECT11027, which is recorded as *H. guilliermondii* by CECT, was identified as *H. opuntiae* in this study.

Table 2
Sequences of oligonucleotide probes labeled with FITC at the 5′ end and the non-labeled competitor used in this study.

Target species	Probe	Position[a]	Sequence (5′–3′)	$\Delta G^{o}_{overall}$[b] (kcal/mol)
C. stellata	Cst-FITC[c]	D133	CTCTATGGCGTTTCTTTC	−11.9
Eight species of *Hanseniaspora*[d]	H8a-FITC	D402	TGAGAGGCCCAAGCCCAC	−15.8
	H8b-FITC	D2	AGGTAATCCCAGTTGGTT	−14.3
Sacch. cerevisiae	H8b-Com[e]	D2	AGGCAATCCCGGTTGGTT	
H. guilliermondii	Hgu-FITC[c]	D506	CAATCCCAGCTAGCAGTAT	−10.0
H. uvarum	Huv-FITC[c]	D507	TCAATCCCGGCTAACAGTA	−9.3
Sacch. cerevisiae	Sce-FITC[c]	D526	TGACTTACGTCGCAGTCC	−13.9
Starm. bacillaris[f]	Sba-FITC	D133	CTCCATGGCGCTCCTTTC	−15.0

[a] Sequence positions refer to the D1/D2 domain of the 26S rRNA gene of *Sacch. cerevisiae* (U44806).
[b] The data rely on the general analysis of mathFISH (Yilmaz et al., 2011) under the same conditions of temperature (46 °C), [Na^+] (0.9 M), and probe concentration (1000 nM).
[c] Probes designed by Xufre et al. (2006).
[d] *H. clermontiae* (sequence accession numbers: AJ512456, AJ512452), *H. guilliermondii* (AB618029, EF449520, U84230), *H. lachancei* (AJ512457, AJ512459), *H. meyeri* (AJ512454, AJ512458, AJ512461), *H. opuntiae* (AJ512453, AJ512451, FM180532), *H. pseudoguilliermondii* (AJ512455, AB525689), *H. uvarum* (EU807899, JX103173, U84229), and *H. valbyensis* (U73596, JQ689026, JN938929).
[e] Oligonucleotide competitor used together with probe H8b-FITC to increase the specificity.
[f] Sequence accession numbers from GenBank database were AY160761, JX103187, EF452193, and EF452215.

species to block the probe hybridization site on the non-target species. Competitor analysis from mathFISH (Yilmaz et al., 2011) was used to evaluate the effect of the competitor. Finally, the probes and the competitor were synthesized (Table 2) and tested first by hybridization with target species, and then, the fluorescence intensity and specificity of each probe were evaluated by FISH tests in pure cultures, mixed cultures, and wine samples using both methodologies, epifluorescence microscopy and flow cytometry. During the entire evaluation process, a multifactor trial of FISH techniques was performed until suitable probe and experimental conditions were found. All probes were labeled with fluorescein isothiocyanate (FITC).

2.3. Specificity test of probes

The specificity of each probe was tested in pure cultures, mixed cultures, and wine samples. All of the samples were collected by centrifugation (1 ml sample, 10,000 rpm for 5 min) and then hybridized separately with each probe. For pure cultures, the cells of each strain in Table 1 were collected directly from their YPD media. Mixed cultures were inoculated by mixing the same order of cells (2–5 × 10^7 cells/ml) from overnight cultures of *Sacch. cerevisiae*, *H. uvarum*, *Starm. bacillaris*, *Torulaspora delbrueckii*, and *Metschnikowia pulcherrima*. Six different wine samples from Macabeo (white) and Garnatxa Negra (red) varieties were prepared in this study. Grapes were picked and fermented in two different vats of the experimental cellar of the Oenology Faculty in Tarragona (Spain) separately, with *Sacch. cerevisiae* inoculated at the beginning. Two wine samples, W and R, were sampled at the end of the two fermentations. Samples MW1 and MR1 were created by adding an *H. uvarum* pure culture at the end of the two fermentations, and MW2 and MR2 were created by adding *Starm. bacillaris* pure cultures. These additions were necessary because no *H. uvarum* or *Starm. bacillaris* was observed at the end of fermentation.

Natural must from the Macabeo variety from our experimental facility was used to validate the probes. After settling, the must was left for 48 h prior to its simultaneous inoculation with 3 different strains of *Sacch. cerevisiae* (1 × 10^6 cells/ml from each strain). Samples were collected from the initial must after settling (time 0), at 12, 48, 60, and 84 h and at the end of fermentation. The samples were plated on YPD (1% yeast extract, 2% peptone, 2% glucose, and 2% Agar, w/v) and 25 colonies were sampled for species identification by sequencing their D1/D2 26S rRNA coding region. The fermentations were performed in triplicates.

2.4. FISH procedure

The multifactor trial for each probe focused on four different parameters: fixation time (1 h, 2 h, 3 h, and overnight), percentage of formamide in the hybridization buffer (0%, 1%, 2%, 5%, 10%, and 20%), hybridization temperature (37 °C, 42 °C, and 46 °C), and time (3 h and overnight), which were evaluated to optimize the hybridization potential of each probe. The effect of each parameter was assessed by performing single-factor tests based on the procedure of Andorrà et al. (2011).

The published FISH procedure used in Andorrà et al. (2011) was adjusted as follows. Collected cells were suspended with 1× phosphate-buffered saline (PBS) and incubated with 4% (v/v) of paraformaldehyde for 1 h at 4 °C and 1000 rpm agitation in a shaker. Fixed cells were centrifuged at 10,000 rpm for 2 min, resuspended with a 1:1 solution of 1× PBS and absolute ethanol, and then stored at −20 °C until required. Approximately 10^6 cells were hybridized at 46 °C for 3 h in 50 μl of hybridization buffer (0.9 M NaCl, 0.01% w/v SDS, 20 mM Tris–HCl, and 1% v/v formamide) with 10 ng/μl of probe and 10 ng/μl of competitor (when it was required). After hybridization, cells were centrifuged at 10,000 rpm for 5 min, resuspended in 100 μl of washing buffer (25 mM Tris/HCl and 0.5 M NaCl), and incubated at 48 °C for 30 min. Then, the cells were centrifuged again, resuspended in 1× PBS, and analyzed immediately if possible but always within 4 h. A Neubauer chamber and epifluorescence microscope (Leica DM4000B, Wetzlar, Germany) equipped with filter I3 were used for cell observation and enumeration.

2.5. Flow cytometry

The fluorescence of the cell suspension in PBS (the same cells indicated in Section 2.4) was quantified using a BD FACSAria III flow cytometer (BD Biosciences, California, USA) equipped with a 15 mW, 488 nm argon-ion laser. Fluorescence was detected using a 530 (±30) nm band pass filter. Daily instrument quality control, including fluorescence standardization, linearity assessment, and spectral compensation were performed to ensure operational consistency from day to day. At least 10,000 events were acquired and recorded in the linear mode for side scatter and in the log mode for fluorescent signals. The data were collected and analyzed using the FACSDiva software (BD Biosciences, California, USA).

2.6. Alcoholic fermentations

Fermentations were performed in triplicate in 50 ml conical tubes filled with 40 ml of natural concentrated must diluted to a sugar concentration of 240 g/l, the common concentration of local grape must (DOQ Priorat). After plating, no colonies were recovered; hence, we considered this must to be sterile. Single fermentations of Sc (*Sacch. cerevisiae*), Hu (*H. uvarum*), and Sb (*Starm. bacillaris*) and mixed fermentations of ScHu (1:1 of *Sacch. cerevisiae* and *H. uvarum*), ScSb (1:1 of *Sacch. cerevisiae* and *Starm. bacillaris*), and ScHuSb (1:1:1 of *Sacch. cerevisiae*, *H. uvarum*, and *Starm. bacillaris*) were performed

separately with 2×10^6 cells/ml of total yeast inocula obtained from overnight YPD cultures. Fermentations were conducted with constant agitation (120 rpm) at two different temperatures, 15 °C and 25 °C, and the entire process was monitored by weight every 12 h until there was no weight loss. Samples (1 ml) were collected at five points (1, 2, 3, 4, and 5) according to the CO_2 releases of 0% (starting point), 25%, 50%, 75%, and 100%, respectively. Cells from each sample were collected after centrifugation at 10,000 rpm for 5 min. Each sample was fixed and then hybridized separately with each species-specific probe. Species were monitored by epifluorescence microscopy and flow cytometry using the same sampling process for both methodologies.

3. Results

3.1. Development of oligonucleotide probes

Four published probes and three new probes were tested in this study (Table 2) to find suitable probes for wine-related yeast species of *Saccharomyces*, *Hanseniaspora*, and *Starmerella*. The published probe Sce-FITC (Xufre et al., 2006) worked well in our initial assays, whereas Hgu-FITC and Cst-FITC (Xufre et al., 2006) exhibited low fluorescence intensities when hybridized with target species and were considered unsuitable.

The three new probes targeting the D1/D2 domain of the 26S rRNA were designed, with two probes (H8a-FITC and H8b-FITC) for eight species of the *Hanseniaspora* genus (*Hanseniaspora clermontiae*, *H. guilliermondii*, *Hanseniaspora lachancei*, *Hanseniaspora meyeri*, *Hanseniaspora opuntiae*, *Hanseniaspora pseudoguilliermondii*, *H. uvarum*, and *Hanseniaspora valbyensis*) and one (Sba-FITC) for *Starm. bacillaris*. H8a-FITC and H8b-FITC were the only two possible probes for *Hanseniaspora* according to an *in silico* analysis. Both probes could hybridize with the eight species of this genus because the D1/D2 sequences of these eight species are closely related phylogenetically (Jindamorakot et al., 2009). However, both probes exhibited some limitations according to the *in silico* analysis: the target site of H8a-FITC incorporated a complex second structure according to the accessibility map; for H8b-FITC, there were only two mismatch bases between the target sequences of this probe and the corresponding sequence of *Sacch. cerevisiae*. The *in silico* analysis was not sufficient for selecting between H8a-FITC and H8b-FITC; thus, both probes were synthesized and tested. H8a-FITC exhibited a low fluorescence intensity when hybridized with *Hanseniaspora* species, while H8b-FITC presented a positive signal when hybridized with *Sacch. cerevisiae*. Because of the high intensity exhibited by H8b-FITC, this probe was selected and a competitor (H8b-Com) was designed to block the hybridization of this probe with *Sacch. cerevisiae*, resulting in the removal of the unspecific signal from *Sacch. cerevisiae*.

Furthermore, after the optimization of the protocol, the results revealed that 1 h of fixation was sufficient for sample preparation. One percent of formamide in the hybridization buffer was necessary for the optimal hybridization efficiency of H8b-FITC, whereas Sce-FITC and Sba-FITC also performed well with 5% and 10% formamide. The results obtained from varying the hybridization temperature and time did not reveal any improvement. However, the percentage of formamide in the hybridization buffer was more critical in determining the hybridization efficiency of H8b-FITC when using the competitor.

3.2. Specificity tests of probes in pure cultures, mixed cultures, and wine samples

For pure cultures, as indicated in Table 1, the probes provided positive signals only when hybridized with strains of the target species. To consider any potential background signal interference from non-target species, a mixed culture was created to provide the same background for all of the species. Cells of *Sacch. cerevisiae*, *H. uvarum*, and *Starm. bacillaris* were distinguished clearly within the mixed yeast cultures by an epifluorescence microscope and flow cytometer (Fig. 1). In the flow cytometry results, the difference between the non-stained cells (FITC less than 10^3) and the stained cells (FITC higher than 10^3) is clear.

The non-hybridized wine samples W and R contained high background fluorescent signals because of the absorption of polyphenol pigments from the wine. However, for all three probes, the fluorescence intensities of the hybridized cells were much higher than the background signal (Fig. 2). The hybridization results of the six wine samples demonstrated that the three target species, *Sacch. cerevisiae*, *H. uvarum*, and *Starm. bacillaris*, can be differentiated in wine samples using both the epifluorescence microscope and the flow cytometer (Fig. 2). This capability was primarily achievable because the fluorescent color from the hybridized target cells differed from the background color and non-target cells in wine and because the target signal was more intense when using the filter for FITC. The H8b-FITC, when coupled with H8b-Com, Sce-FITC and Sba-FITC probes presented high specificity to the target species in the FISH tests performed on pure cultures, mixed cultures, and wine samples.

3.3. Yeast population analysis during alcoholic fermentations

At 25 °C, all of the fermentations with *Sacch. cerevisiae* (pure and mixed fermentations) were completed by 192 h, whereas the pure-culture fermentations with *Starm. bacillaris* (Sb) and *H. uvarum* (Hu) were slower and required 264 h and 336 h, respectively. All of the fermentations were relatively longer at 15 °C: ScHu and ScHuSb terminated after 264 h; ScSb and Sc required 336 h; and Sb and Hu did not complete their fermentations until 336 h. For the pure-culture fermentations, Sb reached point 3 of the fermentation, whereas Hu only reached point 2 (25% CO_2 release). The FISH methodology was used to monitor the yeast population dynamics during the entire fermentation process and the population dynamics are shown in Fig. 3. The population dynamics of *Sacch. cerevisiae* were similar in all of the mixed fermentations, regardless of whether the results were obtained by epifluorescence microscopy or flow cytometry. In contrast, the populations of *H. uvarum* and *Starm. bacillaris* were slightly larger according to the microscopy results compared to those recorded by flow cytometry.

The populations of *Sacch. cerevisiae* and *Starm. bacillaris* in the single fermentations increased from their initial concentration of 10^6 cells/ml to 10^7–10^8 cells/ml, whereas *H. uvarum* grew more slowly with a population level of 10^6–10^7 cells/ml. In all of the mixed fermentations, the *Sacch. cerevisiae* populations increased to 10^7–10^8 cells/ml after inoculation, whereas the populations of *H. uvarum* and *Starm. bacillaris* did not increase and remained at approximately 10^6 cells/ml. The temperature affected the fermentation process: the fermentations at 15 °C were slower than those at 25 °C, while mixed fermentations inoculated with *H. uvarum* were faster than the others at the low temperature. The population dynamics of *Sacch. cerevisiae*, *H. uvarum*, and *Starm. bacillaris* at both 15 °C and 25 °C were similar for both the single and mixed fermentations and did not seem to be affected by the temperature.

While, the fluorescence intensity of the hybridized *Sacch. cerevisiae* cells varied during the fermentation process, those of the *H. uvarum* and *Starm. bacillaris* cells did not. At point 1 of the fermentation, most of the *Sacch. cerevisiae* cells had achieved the highest fluorescence intensity. This intensity declined in the later stages of the fermentations (decreasing by up to 10-fold from the initial fluorescence); however, the *Sacch. cerevisiae* cells always exhibited a higher intensity than the background. These changes in intensity were always observed with the *Sacch. cerevisiae* probes, but the cells within a sample yield did not always produce the same intensity. This result can be observed by the formation of two peaks in the flow cytometry data (Supplementary data).

Finally, the validation of the probes was also tested in a natural fermentation of Macabeo must. As seen in Fig. 4, we detected the presence of *H. uvarum* and *Starm. bacillaris* as the main non-*Saccharomyces* species in the must (approximately 10^6 cfu/ml) as well as traces of

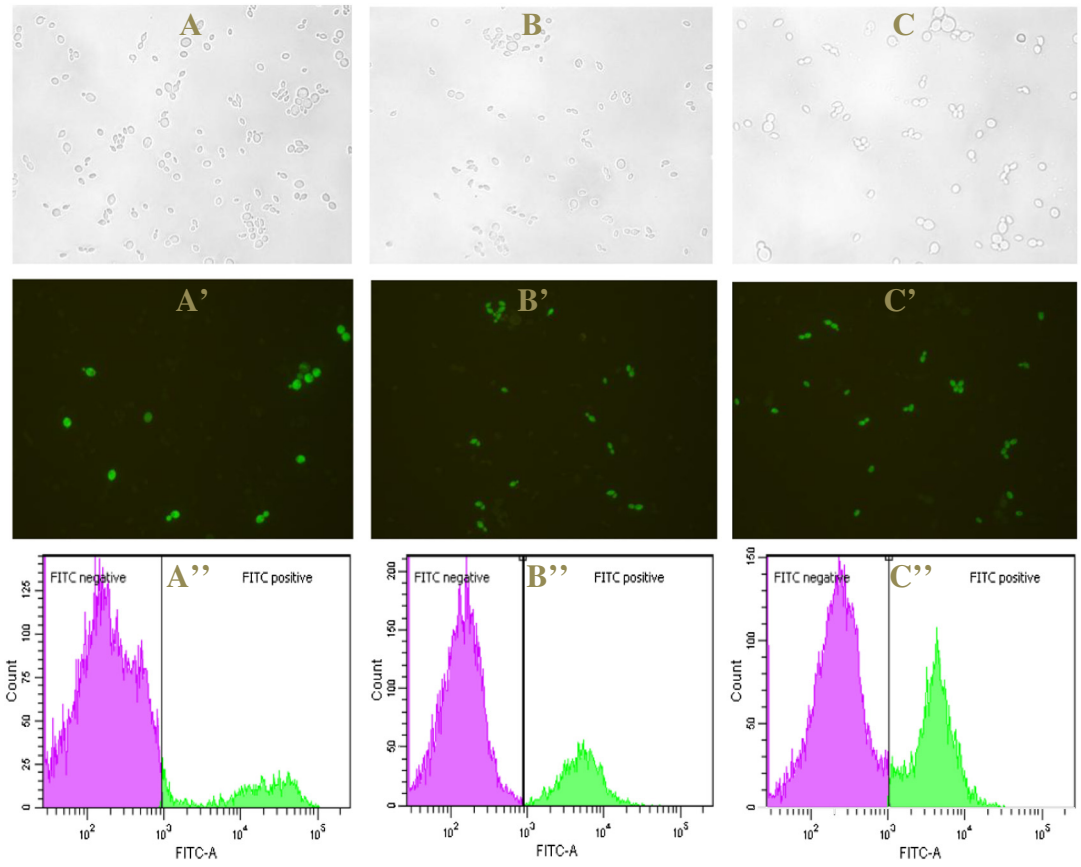

Fig. 1. Fluorescent signal of target species in mixed yeast cultures visualized by microscopy and flow cytometry. Cells hybridized with probes Sce-FITC (A, A′, A″), H8b-FITC coupled with H8b-Com (B, B′, B″), and Sba-FITC (C, C′, C″) and recorded with white light (A, B, C) and filter I3 (A′, B′, C′) of the microscope and by flow cytometry (A″, B″, C″).

T. delbrueckii and *M. pulcherrima* (1 colony each at 0 and 12 h), which disappeared after the inoculation of *Sacch. cerevisiae*. During the start of the alcoholic fermentation, the values obtained on plates and by FISH were very similar. However, at the end of the fermentation, these values differed greatly; *H. uvarum* was not detected by plating, while *Starm. bacillaris* differed by two log units and *S. cerevisiae* by 5-fold. For the total cell count, the values were greater on plates than under the microscope during the initial stages of fermentation and one order of magnitude lower at the end of the fermentation.

4. Discussion

Culture-independent techniques have been developed for the rapid enumeration of yeast populations during the wine-making process, with special emphasis on the determination of both the live and VBNC cells (Andorrà et al., 2010b, 2011; Branco et al., 2012; Cocolin and Mills, 2003; Hierro et al., 2006; Röder et al., 2007). Among them, FISH is a promising technique for detecting target cells directly from wine samples because FISH probes are designed to hybridize with the rRNA inside of cells. In the present study, suitable probes and experimental conditions for detecting *Sacch. cerevisiae*, *Hanseniaspora*, and *Starm. bacillaris* were selected and used for the quantification of each yeast species during the entire fermentation process. Thus, the evaluation of the potential for employing FISH in wine fermentation analyses was the goal of this study. Notably, the yeast cells detected by FISH should be strictly defined as cells with non-degraded rRNA. The stability of rRNA is species dependent (Andorrà et al., 2011); for example, 99% of the 26S rRNA of *Sacch. cerevisiae* was found to be degraded within 24 h after cell death by heat-shock (Hierro et al., 2006).

Early reports have described FISH probes for several wine-related yeast species (Röder et al., 2007; Stender et al., 2001; Xufre et al., 2006); however, only probes for *D. bruxellensis* and *Sacch. cerevisiae* have been successfully used for direct wine sample analyses (Andorrà et al., 2011; Röder et al., 2007). This is the first report of the use of oligonucleotide probes for *Hanseniaspora* and *Starm. bacillaris* in direct wine sample analysis. In addition, the design and evaluation of the oligonucleotide probes followed the important guidelines (regarding specific sequences, accessible sites, and high affinity) suggested by Inácio et al. (2003) and Yilmaz et al. (2011). For example, probes should be designed with −13 to −17 kcal/mol of $\Delta G^{o}_{overall}$ under typical FISH conditions according to principles of the mathFISH program (Yilmaz et al., 2011). However, the $\Delta G^{o}_{overall}$ values of the three probes (Hgu-FITC, Cst-FITC, and Huv-FITC) designed by Xufre et al. (2006) were outside of this recommended range, indicating a low affinity of those probes for target sites, which can most likely explain their low hybridization efficiencies. Unfortunately it could be difficult to achieve a good balance among these guidelines for some yeast species because the possible specific site for specific hybridization (for *Hanseniaspora* species, it was D441–D518) was located in an inaccessible region according to the accessibility map (Inácio et al., 2003). Subsequently, the experimental evaluation provided the final evidence. Furthermore, the probes should be subject to a multifactor trial to evaluate the conditions for the optimal hybridization efficiency. The percentage of formamide in the hybridization buffer is a relevant factor that should be tested for each probe, and mathFISH was demonstrated to be a useful tool with theoretical references for determining the appropriate formamide concentration (Yilmaz et al., 2011).

The high background of the fluorescent signal found in the white and red wine samples was the most likely reason for the limited use of FISH probes for the direct analysis of wine samples in past years. The polyphenols absorbed by yeast walls cause an intensive yellow fluorescence, while the anthocyanins that often form aggregates that include yeast

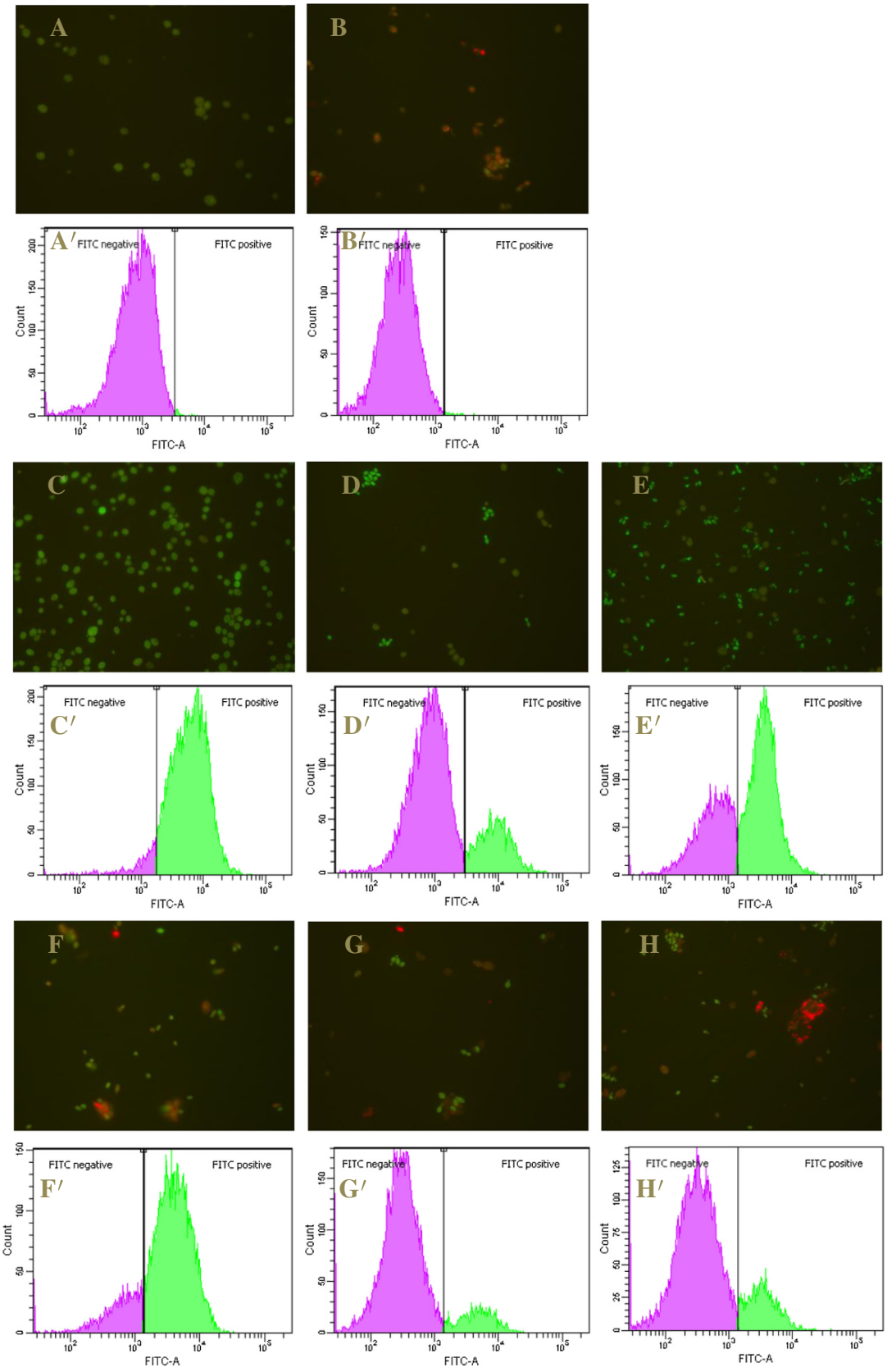

Fig. 2. Fluorescent signals of background and target species in samples W, R, MW1, MW2, MR1, and MR2 visualized by microscopy (A, B, C, D, E, F, G, H) and flow cytometry (A′, B′, C′, D′, E′, F′, G′, H′). Samples W (A, A′) and R (B, B′) without staining. Cells from samples W (C, C′), MW1 (D, D′), MW2 (E, E′), R (F, F′), MR1 (G, G′), and MR2 (H, H′) hybridized with probes Sce-FITC (C, C′, F, F′), H8b-FITC coupled with H8b-Com (D, D′, G, G′), and Sba-FITC (E, E′, H, H′).

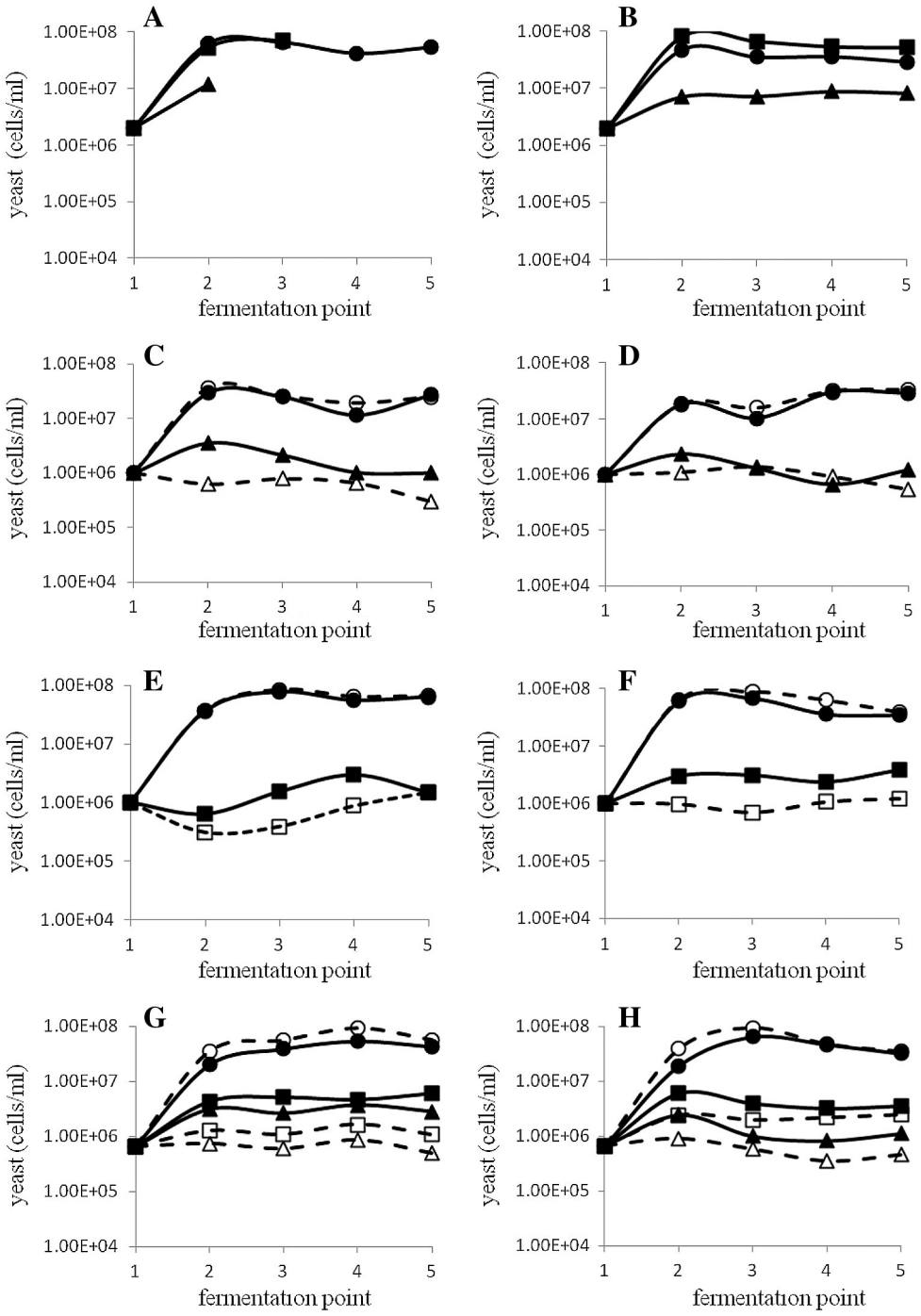

Fig. 3. Yeast population dynamics during alcoholic fermentations at 15 °C (A, C, E, G) and 25 °C (B, D, F, H). Solid lines indicate populations measured by epifluorescence microscopy, and dotted lines indicate populations assessed by flow cytometry (the population quantity shown is the mean of triplicate values). Single fermentations of Sc, Hu, and Sb (A, B). Mixed fermentations of ScHu (C, D), ScSb (E, F), and ScHuSb (G, H). Symbols: (●,○) *Sacch. cerevisiae*; (▲,△) *H. uvarum*; (■,□) *Starm. bacillaris*.

cells cause intense red fluorescence. Thus, probes labeled with fluorochromes of these wavelengths or similar wavelengths (such as the FITC probe used in this study) should be tested for their clear discrimination between the background and the fluorescence of the target.

As proposed in Andorrà et al. (2011), FISH combined with epifluorescence microscopy and flow cytometry was used to monitor the yeast populations during the entire fermentation process. FISH combined with microscopy was useful for analyzing each cell in detail; however, for ecological studies, there is a need for the observation of a large number of cells and a highly experienced operator if the goal is an accurate quantification of the yeast populations present in small numbers. The analysis of a large number of cells under a microscope is tedious and time consuming, and the percentage of cells found in small populations can be biased by the subjectivity of the operator if they specifically look for those minor species. In contrast, the use of FISH combined with flow cytometry is fast, sensitive, and accurate because thousands of cells can be recorded in several seconds. Thus, it is suitable for the high throughput analysis of large numbers of wine samples. For our results, special attention should be paid to the aggregation of cells caused by the fixation step of FISH and this portion of cells should be excluded by gating when using flow cytometry. The aggregation of cells was a common phenomenon observed when using the FISH technique. Fortunately, the aggregation of yeast cells was not as serious as that of bacteria, making FISH combined with flow cytometry easier for yeast.

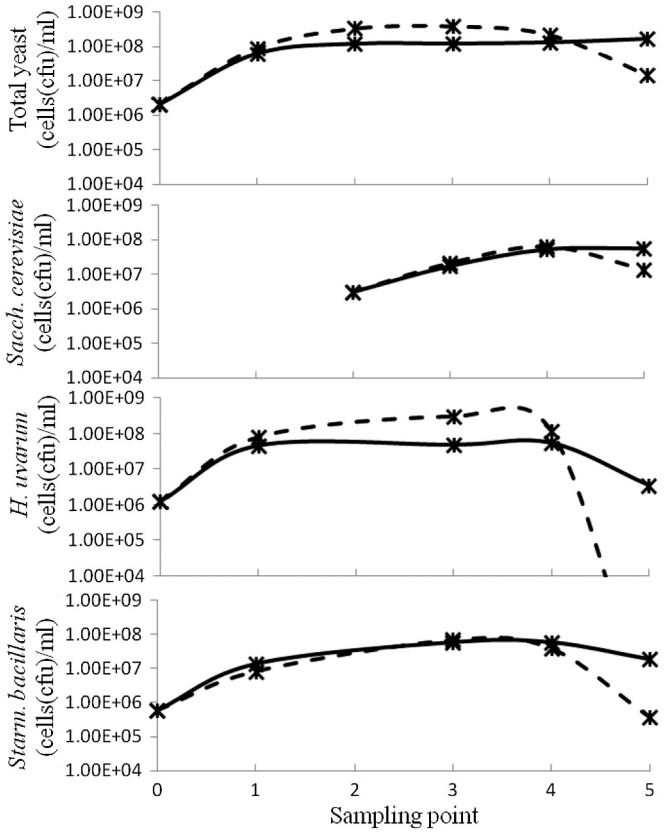

Fig. 4. Yeast population dynamics during the natural fermentation of Macabeo must. Solid lines refer to populations assessed by flow cytometry and dashed lines to plates. Sampling points were: 0, must after settling (density = 1100, 0 h); 1, 12 h; 2, 48 h (time of *Sacch. cerevisiae* inoculation); 3, 60 h; 4, mid-fermentation (density = 1060, 84 h); and 5, final fermentation (density < 1000, 312 h).

Considering the complementary advantages of both methodologies, their combined use would be a better choice for the precise enumeration of yeast populations in wine samples.

Two interesting phenomena concerning yeast population dynamics during wine fermentations were observed in this study. On the one hand, the two non-*Saccharomyces* species maintained their inoculated population sizes in the mixed fermentations, while their populations increase in the single culture fermentations. This result could suggest that the capacity of the non-*Saccharomyces* population to grow was limited and that environmental pressure caused by *Sacch. cerevisiae* might be the primary factor. This finding was also observed in the natural fermentation. Meanwhile, the non-*Saccharomyces* populations did not decrease sharply in the late stages of mixed fermentations as normally observed by culture-dependent methods and as indicated in former ecological studies based on other culture-independent techniques (Andorrà et al., 2010b, 2011; Cocolin and Mills, 2003). Non-*Saccharomyces* yeasts can enter a viable but non-culturable state; under these conditions, they lose their cultivability but maintain their ribosomal RNA (without degradation), and a portion of these cells likely maintain their metabolic activities. However, we cannot completely rule out the possibility that the cells died and their rRNA persisted. In fact, at the end of the natural fermentation, we observed a meaningful difference between the number of cells observed under the microscope and the number of those able to grow on plates, which could have been attributed to either dead cells that maintained their cell structure (and thus their rRNA) or cells that remained alive but were not able to be grown on plates (viable but unculturable). Furthermore, the variation in the fluorescence intensity of the stained *Sacch. cerevisiae* cells during the fermentation, similar to the phenomenon observed by Andorrà et al. (2011), could be related to an increase in ribosome synthesis during the initial stages of alcoholic fermentation in *Sacch. cerevisiae* cells (Novo et al., 2007).

The actual state of the cells detected by FISH was difficult to determine and most likely included VBNC cells, injured cells, and even dead cells with high rRNA stability. Herrero et al. (2006) and Regan et al. (2003) have suggested that the viability of the cells could be assessed using fluorescent dyes such as EMA and propidium iodide (PI) to stain cells with compromised membranes. Branco et al. (2012) reported that direct live/dead staining combined with FISH (LDS–FISH) can be used to effectively assess the viability of *Sacch. cerevisiae* and *H. guilliermondii* during alcoholic fermentation. Thus, the combined use of such dyes and FISH could be evaluated for the detection of the true, viable target yeast population in future work.

In summary, the present study developed specific oligonucleotide probes for *Hanseniaspora* and *Starm. bacillaris*, which were applied in FISH combined with flow cytometry. We were able to directly identify *Sacch. cerevisiae*, *H. uvarum*, and *Starm. bacillaris* in complex wine samples and thus demonstrated the potential for using FISH techniques in wine ecological studies. The design of new probes for other species will help to monitor the population dynamics of various yeast species (including minor populations) during industrial wine fermentations and to detect spoilage yeasts during wine aging and storage. We also demonstrated the successful application of FISH in natural fermentations.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijfoodmicro.2014.08.014.

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