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ORIGINAL RESEARCH ARTICLE

Continuous automated microbial monitoring in laboratory-scale winemaking experiments

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ABSTRACT

The management of microbial flora is a key element of the winemaking process. It impacts process time, fermentation quality (e.g., production of alcohol, development of aroma compounds, absence of undesirable microorganisms) and the overall quality of the final wine product. In microbial flora management, real-time microbial monitoring is crucial. When applying currently used classical methods (e.g., cultivation on plates) or more recent technologies (e.g., flow cytometry), the flora sampling frequency is not optimal and taking around-the-clock measurements involves high labour costs. The objective of this study was to evaluate the feasibility of automated microbial monitoring based on an online flow cytometry system for different laboratory-scale applications in the field of winemaking. Initially, a protocol for automated sampling, double-staining and analysis was validated on yeast and bacterial populations after rehydration of the starter cultures. The system was then tested on a variety of increasingly complex biological systems, simulating its applications in the winemaking process. First, a yeast starter culture preparation for "prise de mousse" was tested. Then, a mixed-culture alcoholic fermentation was monitored. Finally, a microbial-focused observation of wine aging was emulated. By overcoming specific constraints linked to the must medium (e.g., sugars, alcohol contents, production of gas, fermentation duration), the results show the potential of this technology for (1) automated yeast or bacterial monitoring in a wide range of laboratory-scale wine environments, (2) simultaneous monitoring of both total and intact populations of multiple microorganisms, (3) long observation periods, and (4) high sampling frequencies for high-resolution data. It could be particularly useful for facilitating and improving control of potential contaminants or stuck fermentations, as well as better piloting starter preparations, alcoholic fermentations, or malolactic fermentations.

KEYWORDS: automation, flow cytometry, monitoring, wine, yeast, bacteria, real time

INTRODUCTION

Producing wine of a controlled and consistent quality relies heavily on properly managing the development of microorganisms and their subsequent impact on the must (Belda *et al.*, 2017). Monitoring microbial populations (yeasts and bacteria) throughout the entire process (especially alcoholic fermentation and malolactic fermentation) is hence key to ensuring that the process is carried out properly, and thus to guaranteeing the quality of the final wine product. Microbial monitoring is carried out during many stages of the process to ensure the proper implementation of the fermentation starters, avoid the development of unwanted or alteration flora, and monitor and manage the development of mixed cultures over time (Bordet *et al.*, 2020; Ciani *et al.*, 2010; Ciani and Comitini, 2015).

Fermentation is usually monitored either directly by enumerating the microbial population or indirectly by measuring the physico-chemical parameters (e.g., sugars, CO_2). These latter parameters can be monitored online, but the continuous monitoring of wine microorganisms in a more direct way has not yet been implemented in cellars. Traditional microbial techniques are already used for this purpose, but they have multiple shortcomings that limit monitoring: they are labour-intensive (e.g., microscopy), the results are obtained with a significant delay (e.g., culture on Petri dish) and the flora cannot be identified (e.g., OD measurements). Recently, flow cytometry has allowed researchers to reduce most of these shortcomings (Longin *et al.*, 2017). Flow cytometry is a rapid, cultivation-independent method, capable of single-cell resolution and hence of differentiating subpopulations of yeasts and bacteria. Despite being a fast measurement technology, flow cytometry still requires manual labour, especially for sampling, diluting and staining, which limits measurement frequency and makes around-the-clock monitoring challenging.

The automation of flow cytometry can overcome these limitations and enable autonomous sample collection, staining and analysis. Samples are collected from the studied medium at regular intervals, then mixed with a staining solution, incubated at appropriate temperature, and finally pumped into a flow cytometer for analysis. Such systems have been used to monitor water quality (Besmer *et al.*, 2014) and bioreactor processes (Heins *et al.*, 2022). Other studies include automated monitoring involving fermentations and/or use of *Saccharomyces cerevisiae* (Abu-Absi *et al.*, 2003; Freitas *et al.*, 2013; Kacmar *et al.*, 2004). In the field of winemaking, however, no studies on the application of an automated system yet exist.

The aim of this work was to study the feasibility of using online microbial monitoring for various laboratory-scale applications relevant to winemaking, namely: (1) the rehydration of active dried microorganisms (yeasts, bacteria), (2) the adaptation of active dried yeasts before inoculation in wine, (3) the monitoring of alcoholic fermentation, and (4) the tracking of microbial populations during wine aging.

MATERIALS AND METHODS

1. Monitoring of microbial populations by automated flow cytometry

In all the experiments, microbial monitoring was implemented according to the following workflow: (1) sample collection at regular intervals, (2) dilution (if applicable), (3) staining and incubation, (4) measurement by flow cytometry, and (5) data processing and analysis.

An onCyt OC-300 automation unit (onCyt Microbiology AG, Dübendorf, Switzerland) was used (see diagram in Figure S1) as the interface between sample containers and the flow cytometer. The system was programmed to collect the sample automatically from the medium at intervals; the interval was 25 min, except when monitoring wine aging, for which the samples were taken every 12 h. Before each sampling, all fluidic parts of the automation system were automatically cleaned with a sodium hypochlorite solution (1 % active chlorine) and then a sodium thiosulfate solution (50 mM), followed by rinsing with ultrapure water to avoid contamination from the previous sample. Before each measurement, the complete dead volume of the sample inside the sample tubing was replaced (575 µl). Once retrieved, the samples were used undiluted or diluted 10-fold, 100-fold or 1000-fold in TRIS buffer (10 mM, pH 8) depending on the expected concentrations in a given experiment (see Materials and methods, Section 2.). The sample was then stained twice within 2 min to apply multiple staining assays (see below). Assuming that no substantial change to the sample had occurred within this short period, the resulting concentrations from both assays were interpreted as belonging to the same time point.

The diluted samples were then stained with an established assay using SYBR Green I (SG) stain and a mix of SG and Propidium Iodide (PI) stains respectively (Barbesti *et al.*, 2000; Gatza *et al.*, 2013; Nescerecka *et al.*, 2016, Sizzano *et al.*, 2022). SG specifically binds to nucleic acids and in this state emits strong green fluorescence signals upon excitation by a blue laser (488 nm). As SG can enter all cells regardless of their biological state, all particles with high green fluorescence signals in the absence of a PI stain are considered as the "total" population. PI also specifically binds to nucleic acids, but it emits stronger red fluorescence signals upon excitation by a blue laser (488 nm). PI can only enter cells with compromised membranes, where it will occupy nucleic acid binding sites instead of SG, resulting in lower green fluorescence but higher red fluorescence. Hence, all particles that retain high green fluorescence signals caused by SG, despite the presence of a PI stain, are considered as the "intact" population. In contrast, the intact population subtracted from the total population quantifies the "damaged" or "dead" cells, which had shifted into the signal space of low green and high red fluorescence (Longin *et al.*, 2017). In the case of mixed fermentation (see Materials and methods, Section 2.3.), only PI staining was applied (no SG), as yeasts can be detected as a result of their autofluorescence or the fluorescence related to the presence of a GFP protein.

SG and PI (Invitrogen™, Thermo Fisher Scientific Inc., Waltham, MA, USA) were prepared at a concentration of 3.92 and 24 µmol/L respectively in sterile TRIS buffer (10 mM, pH 8) to obtain 2X staining solutions to be used within the automated system and mixed with the sample at a ratio of 1:1. The stained samples were incubated for 10 min at 37 °C before being pumped to the flow cytometer.

A BD Accuri™ C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) was used with the following settings: analysed volume of 20 µL, flow rate of 34 µL/min, excitation by a 488 nm wavelength argon laser, detection by the FL1 long-pass filter (530 nm) for green fluorescence and the FL3 long-pass filter (670 nm) for red fluorescence. The trigger was set on FSC for yeast analysis, with a FSC-H threshold of 80,000. For bacteria-related analyses (bacteria after rehydration and wine during aging), the trigger was set on FL1, with FSC-H and FL1-H thresholds of 10,000 and 600 respectively. A summary of these threshold parameters can be found in Table S1.

Data were analysed using the cyPlot v3.08 software (onCyt Microbiology AG, Dübendorf, Switzerland) for the quantitative analysis, and the BD Accuri C6 software for the qualitative evaluations. Fixed gates were defined for each type of experiment (bacteria with SG+PI, yeasts with SG+PI, yeasts with GFP+PI) to discriminate the microbial populations from background signals and differentiate between the aforementioned total, intact and damaged populations. Summary statistics for gated FCM files were then exported to .csv files and population concentration calculated based on dilution level used for each sample.

2. Studied biological systems

2.1. Active dried yeasts and bacteria after rehydration

Dried active yeasts *Saccharomyces cerevisiae* 18-2007 from IOC (Épernay, France), which are often used as a fermentation starter, were investigated. Dried yeasts (1 g) were rehydrated in 100 mL of physiological water at 37 °C. The yeast suspension was placed on a Variomag (Port Orange, FL, USA) shaking table (150 rpm). Monitoring of microbial population was started after 30 min of homogenisation and lasted for 5 h. The automated flow cytometry system was set to dilute the samples 100-fold before staining. Two flasks were monitored in parallel.

Lactic bacteria involved in malolactic fermentation (*Oenococcus oeni*, "Maxiflore Elite" from IOC) were used. Dried active bacteria (1 g) were rehydrated in 100 mL of an activation solution (based on inactivated yeasts) at 20 °C. The bacterial suspension was placed on a Variomag shaking table (150 rpm). The monitoring of the microbial population started after 30 min of homogenisation and lasted for 25 h at 20 °C. The automated flow cytometry system was set to dilute the samples 100-fold before staining. Three biological replicates were monitored in parallel.

2.2. Yeast starter culture for "prise de mousse"

Dried active yeasts *Saccharomyces cerevisiae* 18-2007 from IOC were investigated. Dried yeasts (1 g) were first rehydrated in 100 mL of physiological water at 37 °C. The yeast suspension was placed on a Variomag shaking table (150 rpm) for 15 min.

The first step of the preparation of the yeast starter culture was an adaptation of the yeast to a "wine environment" in a medium containing high sugar concentration and alcohol. The yeast suspension was mixed with 80 mL of sucrose syrup (600 g/L) and 100 mL of wine, resulting in a final volume of 280 mL. This wine was obtained from the alcoholic fermentation of a white grape juice (standardised by sucrose to up to 200 g/L of total sugars) by the same yeast strain. Then 100 mg/L of nitrogen source (ammonium sulfate) was added. Media components were purchased from Sigma™ (Merck KGaA, Darmstadt, Germany). The mixture was kept homogenised at 20 °C in a sterile 500 mL Erlenmeyer flask and monitored until the density had dropped to 1.030 (measurement with an DMA 35 density meter (Anton Paar GmbH, Graz, Austria)). The automated flow cytometry system was set to dilute the samples 100-fold before staining. Three biological replicates were monitored in parallel.

Subsequently, the second step of preparation of the yeast starter culture was made in a medium allowing adaptation and growth. A 56-mL aliquot of the previous culture (adaptation medium) was used to inoculate a wine medium composed of 624 mL of wine, 160 mL of water and 160 mL of sucrose syrup, resulting in a final volume of 1 L. To this 150 mg/L of nitrogen source (ammonium sulfate) was added. This medium was kept homogenised at 20 °C in a sterile 1 L Erlenmeyer flask and monitored until the density had dropped and stabilised. The automated flow cytometry system was initially set to dilute the samples 10-fold, then it was set to dilute the sample 100-fold after 49 h, when the events rate had reached values too high for reliable measurement by the cytometer (> 1000 events·µL^{-1}). Three biological replicates were monitored in parallel.

2.3. Mixed culture of two yeasts during alcoholic fermentation

Two yeast species, well known for their importance in must fermentation, were investigated in this study: *Saccharomyces cerevisiae* 59A HO::eGFP (*Sc*), a haploid derivative of the commercial wine strain EC1118 (Lallemand Inc., Montréal, QC, Canada) and *Lachancea thermotolerans* BBMCZ7FA20 (*Lt*) (previously isolated and identified by Sadoudi *et al.* (Sadoudi *et al.*, 2012)). The eGFP mutation in *S. cerevisiae* confers a green fluorescence to this strain and allows two yeast species to be differentiated by flow cytometry. The mutant strain has similar characteristics (growth and oenological kinetics) to the commercial strain it is derived from (unpublished data). Yeasts were preserved in YPD/glycerol (50/50 w/w) at -80 °C in the laboratory collection.

Each strain was first grown at 28 °C on YPD agar (glucose 20 g/L, peptone 10 g/L, yeast extract 5 g/L, agar 18 g/L). Then a preculture was prepared in MS300 liquid medium at 28 °C and agitated at 150 rpm. This synthetic must was first developed by Bely *et al.* (Bely *et al.*, 1990) and is commonly used in oenological research (Alonso-del-Real *et al.*, 2019;

Taillandier *et al.*, 2014; Vendramini *et al.*, 2017; Zupan *et al.*, 2013). It contains carbon sources (glucose, fructose, organic acids), nitrogen sources (mineral and organic, 300 mg/L), minerals, vitamins, and other growth factors. Media components were purchased from Sigma™. The yeasts were collected at the end of the exponential phase to inoculate the culture media.

Sterile glass flasks (Schott AG, Mainz, Germany) (GL45 screw thread, effective volume 1 L) were filled with 1 L of synthetic must. Media were inoculated with 10^6 cells/mL of *L. thermotolerans* and 10^6 cells/mL of *S. cerevisiae*. Cultures were carried out in these non-hermetically closed Schott flasks, and kept homogenised at a temperature of 20 °C. The automated flow cytometry system was first set to dilute the samples 10-fold, then the dilution factor was increased with increasing population growth. Three biological replicates were monitored in parallel.

2.4. Wine during aging

The monitoring of microorganisms was performed every 12 h for 13 days on the natural flora of 200 mL of red wine from a bottle produced by the University of Burgundy, which was kept homogenised at 20 °C. Only one 500 mL flask was monitored and kept open to encourage natural inoculation and simulate contamination. The wine was not filtered before sampling and was not prediluted before staining; however, there is an inherent dilution in the staining process to a factor of x2 due to the mixing of the sample with the stain solution (see Materials and Methods, Section 1).

RESULTS

The results are divided into four parts. The first step was to validate the chosen automation and detection system for both bacteria and yeast enumeration, differentiation and viability characterisation (Part 1). In the second step, the system was used for monitoring biological systems simulating various applications in wine production: a yeast inoculum for sparkling wine (Part 2), a mixed culture alcoholic fermentation (Part 3), and a red wine during aging (Part 4).

1. Tuning and validation of methods for automated monitoring of wine microorganisms

The first step was to validate the feasibility of automatically monitoring the essential microorganisms in wine production: the yeast *Saccharomyces cerevisiae*, the main microorganism involved in alcoholic fermentation, and the bacterium *Oenococcus oeni* involved in malolactic fermentation.

Preliminary experiments (data not shown) allowed us to determine the optimal values linked to the following components of the automated analysis by flow cytometry: (1) sampling volume, (2) sampling frequency, (3) sample preparation (dilution, staining, mixing, incubation) based on previously published studies (Besmer *et al.*, 2014, Prest *et al.*, 2021, Joran *et al.,* 2022). It was also necessary to validate the method of data analysis. To this end, a simple and short analysis of post-rehydration suspensions of microorganisms in physiological water was conducted.

First of all, the feasibility of detecting and enumerating the total and intact population of *Saccharomyces cerevisiae* by the automated system was investigated.

The density plots in Figure 1 show the signals corresponding to green and red fluorescence (FL1-A and FL3-A axis respectively) for each event. The "SG +" gate was chosen to select the yeast population labeled by SG. When the sample was stained with SG only (Figure 1A), the "SG +" gate corresponded to the total population. When double staining was carried out (SG and PI, Figure 1B),

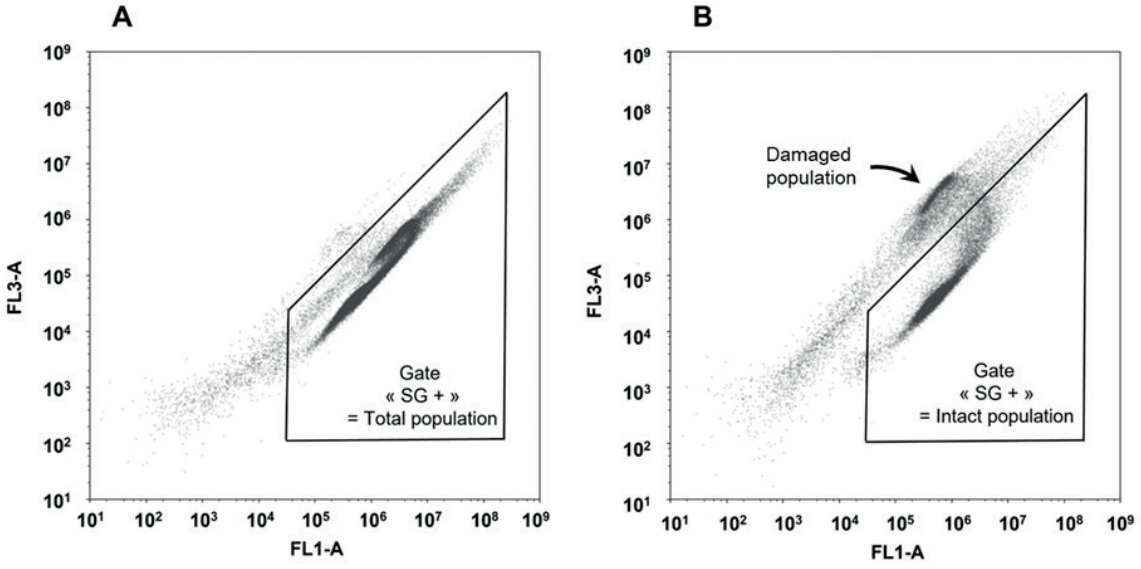


FIGURE 1. Exemplary density plots (t = 3h45) obtained with automated flow cytometry during the monitoring of rehydrated yeast (*Saccharomyces cerevisiae*) after staining with only SG (A) and both SG and PI (B).

Each dot represents the fluorescence intensity of a given particle in the green fluorescence range (x-axis, FL1-A, logarithmic) and the red fluorescence range (y-axis, FL3-A, logarithmic). The "SG +" gate delineates the microbial population (yeast cells in this experiment) labeled by SG. This corresponds to the total population for SG staining only and to the intact population for SG-PI staining.

the "SG +" gate corresponded to the intact population. The damaged cells increased in red fluorescence and decreased in green fluorescence due to binding by PI, leading to a shift out of the gate. Some of these cells are visible above and to the left outside the gate in Figure 1B.

The density plots in Figure 1 show a clear separation of the stained cells and the background in the case of SG-staining (Figure 1A) and of the intact cells and damaged cells in that of SG-PI-staining (Figure 1B). Only marginal numbers of events were located at the border of the gates for the quantification of cells. Such clear separation is needed to ensure the reliability and representativeness of the calculated cell concentrations. This was observed for all measurements over the course of the experiment and allowed total and intact yeast population to be monitored, as depicted in Figure 2.

The yeast concentrations in Figure 2 show that the automated flow cytometry system produces stable and repeatable results for both total and intact cell concentration (1.1×10^8 and 5.5×10^7 cell/mL) over the course of 5 h. The variation coefficient between all collected samples is 5.3 % and 8.0 % for intact and total population respectively. Even when assuming the biological population was constant over the course of the experiment and thus inputting this variation solely to the machine, these values are acceptable and validate the system's reliability. The fraction of the intact population in the total population is about 55 % after rehydration, which is comparable to values obtained previously in similar conditions with non-automated flow cytometry (Attfield *et al.*, 2000).

The same protocol was applied to a bacterial population, as in winemaking bacterial inoculation is often carried out to control the start and the reproducibility of malolactic fermentation. The population of *Oenococcus oeni* was monitored using the automated flow cytometry system.

As presented in the cytogram in Figure 3, a large amount of background noise (event clouds outside the "SG +" gate in Figure 3A) was observed in this case. Based on blank measurements of the rehydration medium, it was possible to show that these background signals were due to the activation solution (containing inactivated yeasts) whose presence in the rehydration medium is necessary for proper rehydration of *Oenococcus oeni*. The "SG +" gate accounts for the total bacteria population when they are stained with only SG (Figure 3A), and for the intact population when a double staining SG - PI is used (Figure 3B). Similar to the monitoring of yeasts (see Figure 1), some of the damaged cells that took up PI and thus increased in red fluorescence and decreased in green fluorescence can be seen above and to the left outside the gate.

A clear separation of stained cells and background was visible in the case of SG-staining (Figure 3A) and of intact cells, damaged cells and background in that of SG-PI-staining (Figure 3B). Only marginal numbers of events were located at the border of the gates for the quantification of the cells. This clear separation is particularly needed in samples containing higher concentrations of background particles. This was observed for all measurements during the course of the experiment and allowed the total and intact bacterial population to be monitored, as depicted in Figure 4.

As can be seen in Figure 4, the automated system monitored the total bacterial population during the period of the experiment (25 h). There was little dispersion between the

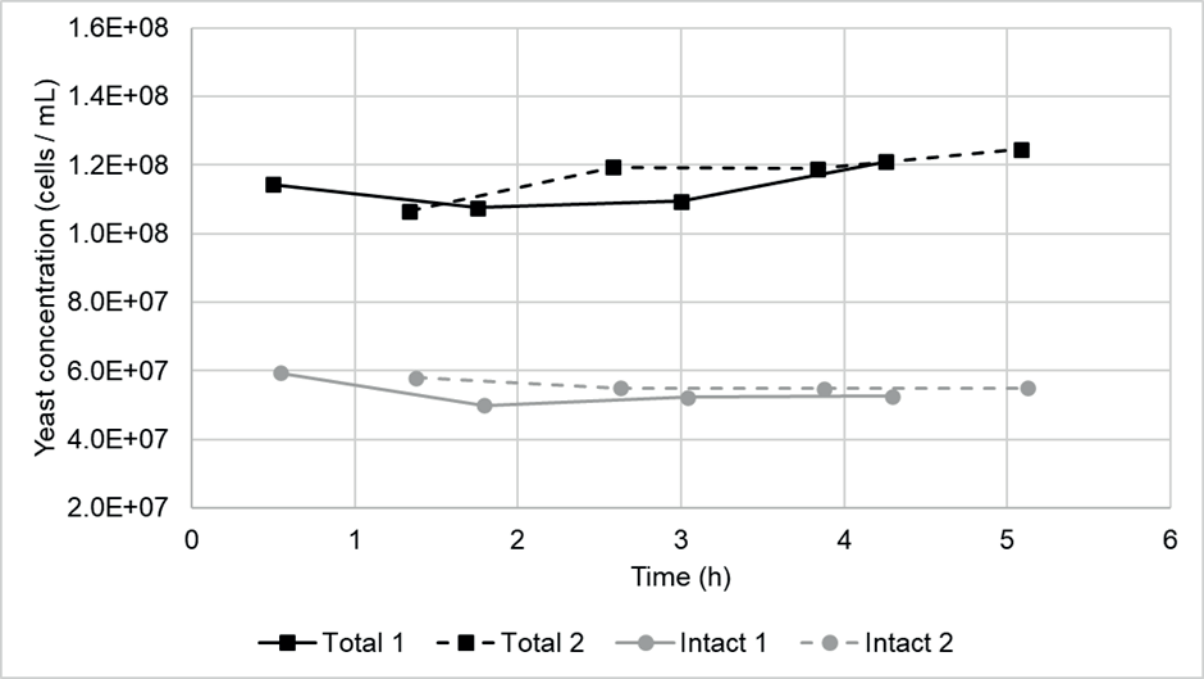


Figure 2. Total and intact yeast concentration of a fermentation starter (*Saccharomyces cerevisiae*), monitored by automated flow cytometry for 5 h

Black squares and grey circles represent respectively total and intact yeast concentration. Continuous and dashed lines correspond to two biological replicates. For each replicate, a measurement was taken every 75 min. t = 0 h indicates the start of rehydration.

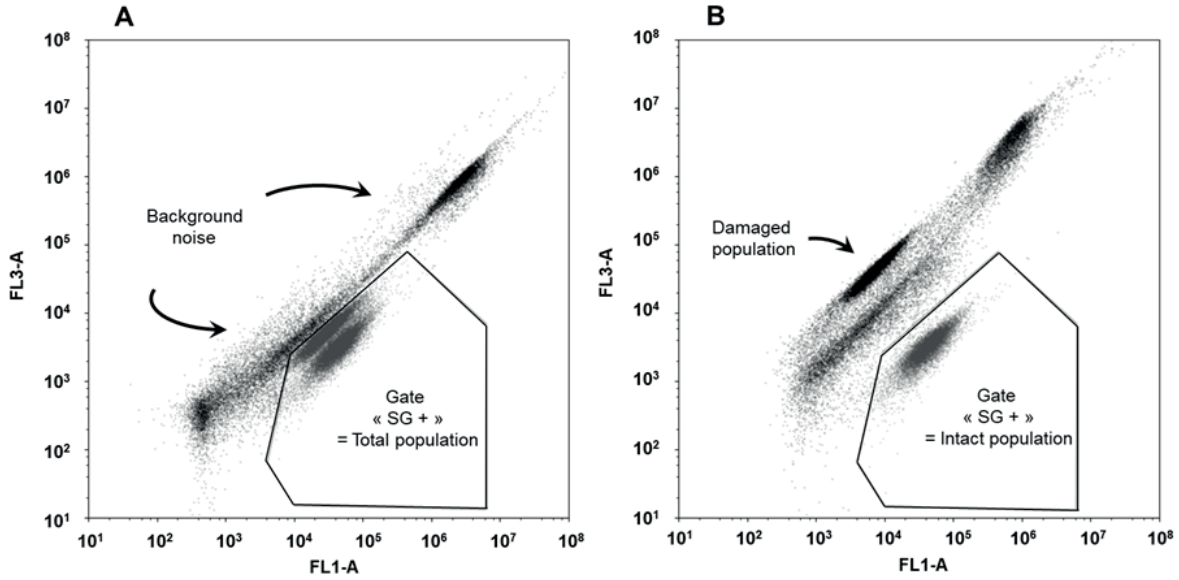


FIGURE 3. Exemplary density plots (t = 3h00) obtained with automated flow cytometry during monitoring of rehydrated bacteria (*Oenococcus oeni*) after staining with only SG (A) and both SG and PI (B).

Each dot represents the fluorescence intensity of a given particle in the green fluorescence range (x-axis, FL1-A, logarithmic) and the red fluorescence range (y-axis, FL3-A, logarithmic). The "SG +" gate delineates the microbial population (bacterial cells in this experiment) labeled by SG. This corresponds to the total population for SG staining only and to the intact population for SG-PI staining.

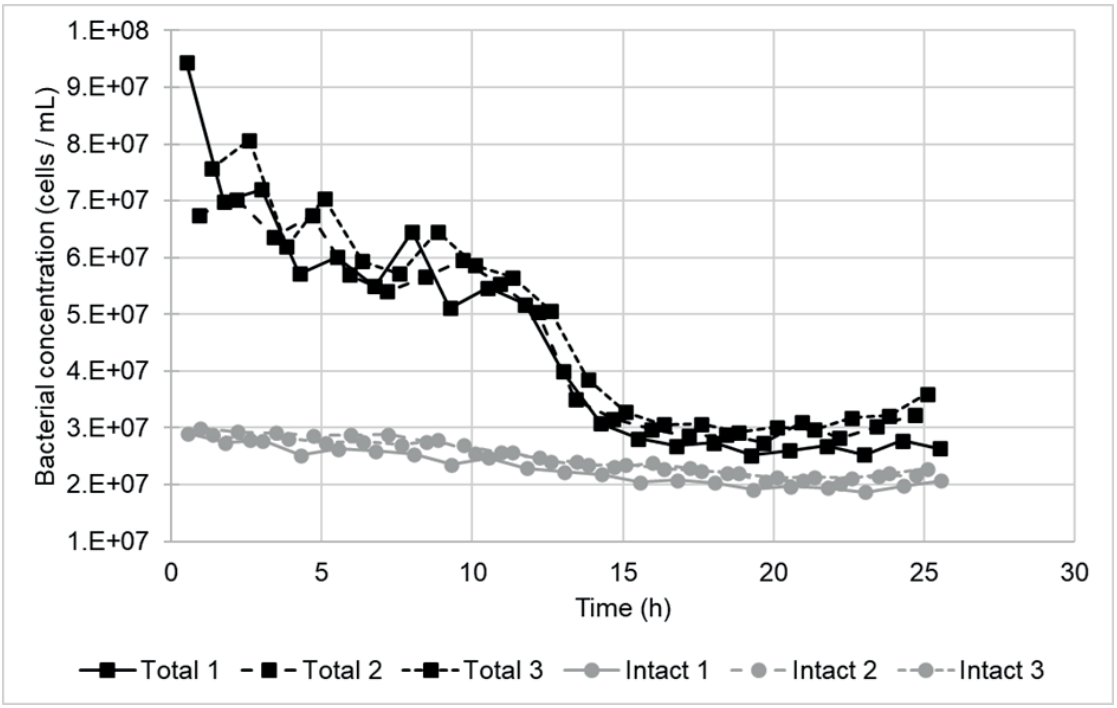


FIGURE 4. Total and intact bacterial concentration of a fermentation starter (*Oenococcus oeni*), monitored by automated flow cytometry for 25 h.

Black squares and grey circles represent total and intact yeast concentration respectively. Continuous and dashed lines correspond to three different biological replicates. For each replicate, a measurement was taken every 85 min. t = 0 h indicates the start of rehydration.

results of the three biological replicates: the average variation coefficient of the replicates was 4.8 % and 7.3 % for the intact and total populations respectively. The fraction of the intact population in the total population was about 40 %, a common viability value for *Oenococcus oeni* after rehydration (Zhao and Zhang, 2005). The total bacterial concentration, initially around 8 x 10^7 cells/mL, decreased during the experiment and stabilised after 15 h at about 3 x 10^7 cells/mL. The intact bacterial concentration was more stable, being between 2 x 10^7 and 3 x 10^7 cells/mL.

2. Microbial monitoring during starter culture preparation for "prise de mousse"

After having shown that the automated flow cytometry system can monitor a yeast population in a simple medium (physiological water), the next step was to apply it to a real winemaking process: the preparation of a yeast inoculum for the "prise de mousse" stage of sparkling wine. This preparation comprises a two-step process with first an adaptation phase of the yeasts inoculated in a medium containing sugar and alcohol, and then a transfer of these yeasts to a second sugar- and alcohol-rich adaptation medium, in which the cells will start to grow.

During the adaptation step, basic yeast monitoring by automated flow cytometry was carried out over several days of minimal growth in order to validate the reliability of the system over longer periods of time. It also constituted the first test of sampling a more complex medium than physiological water (high sugar content, wine, production of CO_2).

At the beginning of the adaptation step, the initial yeast concentration measured by the automated system was about 4 x 10^7 cells/mL (Figure 5); this was in line with the concentration of the dehydrated yeast and the dilutions used for the preparation. During the adaptation step, the total and intact population increased slightly. There was little variability between the three biological replicates (average variation coefficient between replicates of 5.1 % and 6.1 % for the total and intact populations respectively), thus confirming that results from the three sampling lines did not drift apart during the experimental process due to potential technical issues, such as fouling by yeast accumulation or tubing deformation. This experiment lasted about 3 days without outputting any outlier values, indicating that the clean-in-place system and protocol are efficient.

These results showed that the studied system is able to automatically monitor total and intact yeast populations in a complex medium containing sugar and alcohol over several days without drift. The system could thus be used for the following experiments, which involved monitoring for even longer periods of time, and, as yeast growth occurred (albeit quite slowly), aimed to establish if a mid-experiment change in dilution level could significantly impact the results.

At the beginning of this growth step, the total yeast population measured by the automated flow cytometry system was 2.2 x 10^6 cells/mL, which was as expected given the dilutions used for the preparation. Very low variability was observed between the three biological repetitions (average variation coefficient of the replicates were 7.7 % and 8.0 % for the total and intact populations respectively), but the very first

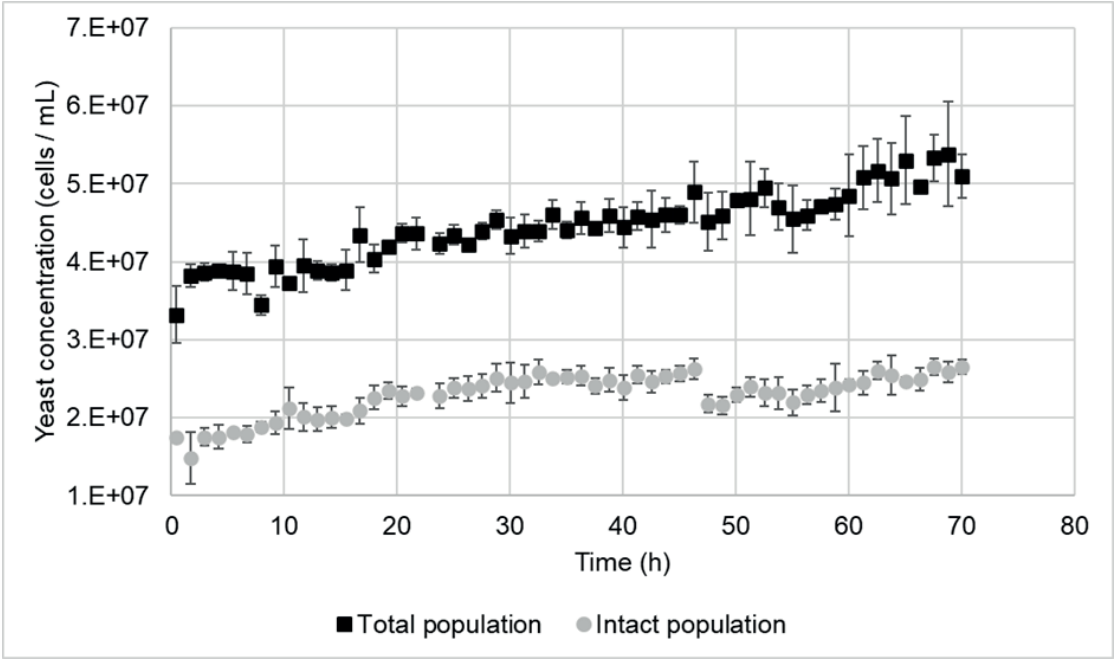


FIGURE 5. Total and intact yeast concentration (*Saccharomyces cerevisiae*) during adaptation phase, monitored by automated flow cytometry.

Black squares and grey circles represent total and intact yeast concentration respectively. Each square and circle represents the mean of 3 biological replicates. For each replicate, a measurement was taken every 85 min.

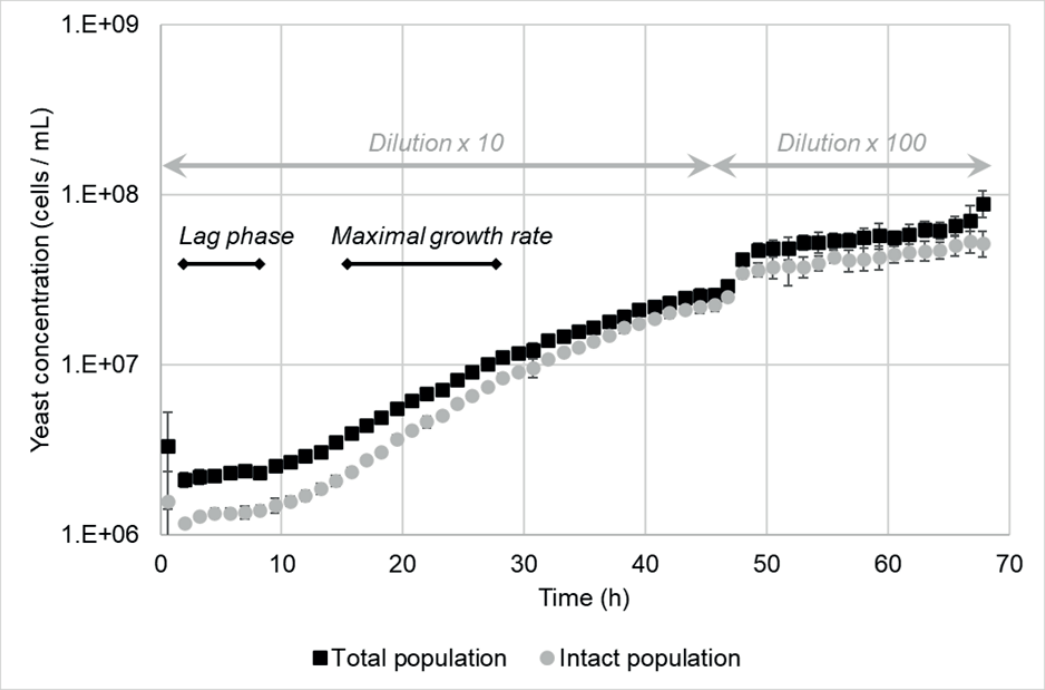


FIGURE 6. Total and intact yeast concentration (*Saccharomyces cerevisiae*) during the growth step in sugar- and ethanol-rich medium, monitored by automated flow cytometry.

Black squares and grey circles represent total and intact yeast concentration respectively. Each square and circle represents the mean of 3 biological replicates. For each replicate, a measurement was taken every 85 min.

data point shows instability (its variation coefficients being above 50 %) and can be considered an outlier. The system was able to monitor the growth of total and intact population for the whole growth step (3 days), until a total population of about 9 x 10^7 cells/mL was reached. The high-frequency monitoring allowed the different growth phases to be determined precisely and accurately. After a lag phase of about 8 h at a concentration of about 1.3 x 10^6 cells/mL, the intact population increased at a maximal growth rate (μ_{max}) of 0.10 cells/h (see Supplementary data, Figure S3), and seemed to have reached the stationary phase by the end of the experiment.

After 47 h, the dilution of the sample was switched from 10-fold to 100-fold. This caused a jump in concentration calculated from the actual measurements and the nominal dilution factor. This suggests that the two dilution regimes do not work equally well.

3. Monitoring of two different yeast populations during mixed culture alcoholic fermentation

This experiment had two objectives. First, it aimed to determine if the system could be used for the complete and proper monitoring of one of the most studied process steps in wine research: alcoholic fermentation.

Second, as mixed culture fermentations constitute a highly studied subject in today's literature, it was also of interest to determine whether the system would allow two different yeast species to be properly differentiated over time, as increasing acquisition frequency is both very valuable and quite challenging for studies on this subject.

Thus, the automated flow cytometry system was used for monitoring a mixed culture alcoholic fermentation involving two yeasts: a mutant GFP+ of *Saccharomyces cerevisiae* and *Lachancea thermotolerans*.

As can be observed in the density plots (Figure 7), PI staining revealed the "damaged" yeasts via the FL3 channel. As regards the intact cells, the "GFP -" and "GFP +" gates were used to estimate the *L. thermotolerans* population and the *S. cerevisiae* population respectively.

These results show that it is possible to monitor the population of two different yeasts at the same time throughout the duration of the fermentation by automated flow cytometry (see Figure 8). The system gave repeatable results for more than 5 days (average variation coefficient between replicates of 8.3 and 16.2 % for *Sc* and *Lt* respectively). By carrying out automated monitoring at the frequency of sampling applied here, it was also possible to precisely determine the different growth phases of both yeasts. For *Saccharomyces cerevisiae*, after a lag phase of about 8 h at a concentration of 4 x 10^6 cells/mL, the intact population increased with a maximum growth rate (see Supplementary data, Figure S4) (μ_{max}) of 0.22 cells/h, before reaching the stationary phase at about 32 h and stabilising at 2 x 10^8 cells/mL. The growth of *Lachancea thermotolerans* was faster at the beginning (lag phase of only 3 h, μ_{max} of 0.35 cells/h), but it reached a maximum of 3 x 10^7 cells/mL more quickly (after 16 h) (most probably because of interactions resulting from *S. cerevisiae* development, as seen in previous studies (Comitini *et al.*, 2011; Fairbairn *et al.*, 2021; Gobbi *et al.*, 2013; Kemsawasd *et al.*, 2015)), and the population tended to decrease until the end of the experiment.

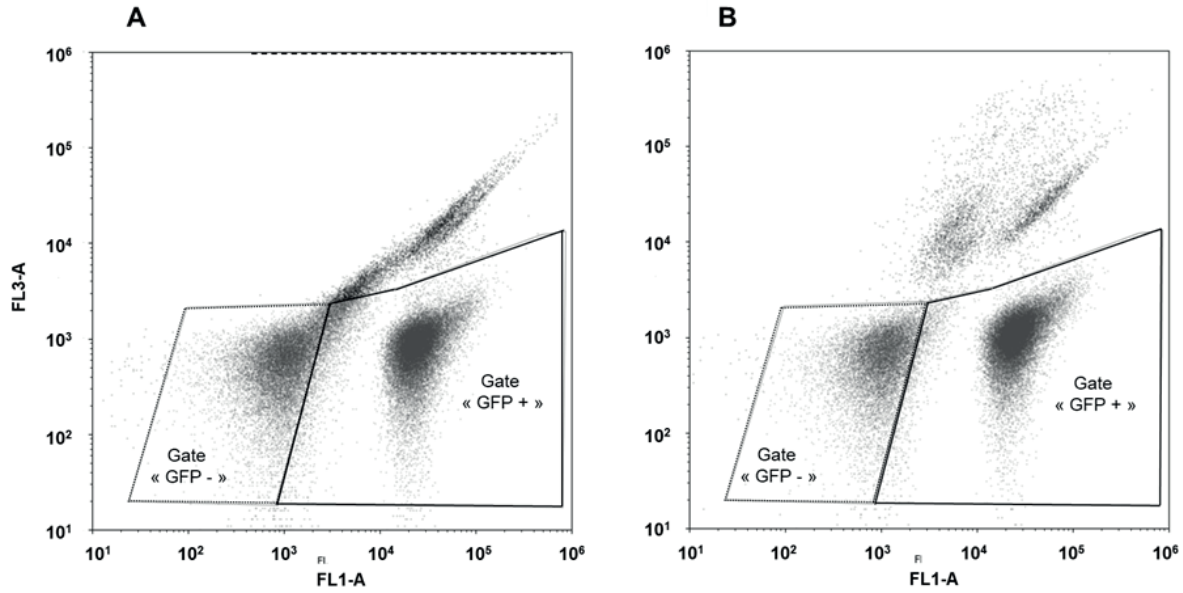


FIGURE 7. Exemplary density plots (t = 22h00) obtained with automated flow cytometry during monitoring of a mixed culture alcoholic fermentation involving *Saccharomyces cerevisiae* and *Lachancea thermotolerans* without staining SG (A) and after staining with PI (B).

Each dot represents the fluorescence intensity of a given particle in the green fluorescence range (x-axis, FL1-A, logarithmic) and the red fluorescence range (y-axis, FL3-A, logarithmic). The "GFP +" and "GFP -" gates delineate the yeast population emitting more green fluorescence because of the presence of the GFP protein (*S. cerevisiae*) or no specific fluorescence (*L. thermotolerans*) respectively. Some of the damaged cells that take up PI and thus increase in red fluorescence and decrease in green fluorescence can be seen above and to the left outside the gate.

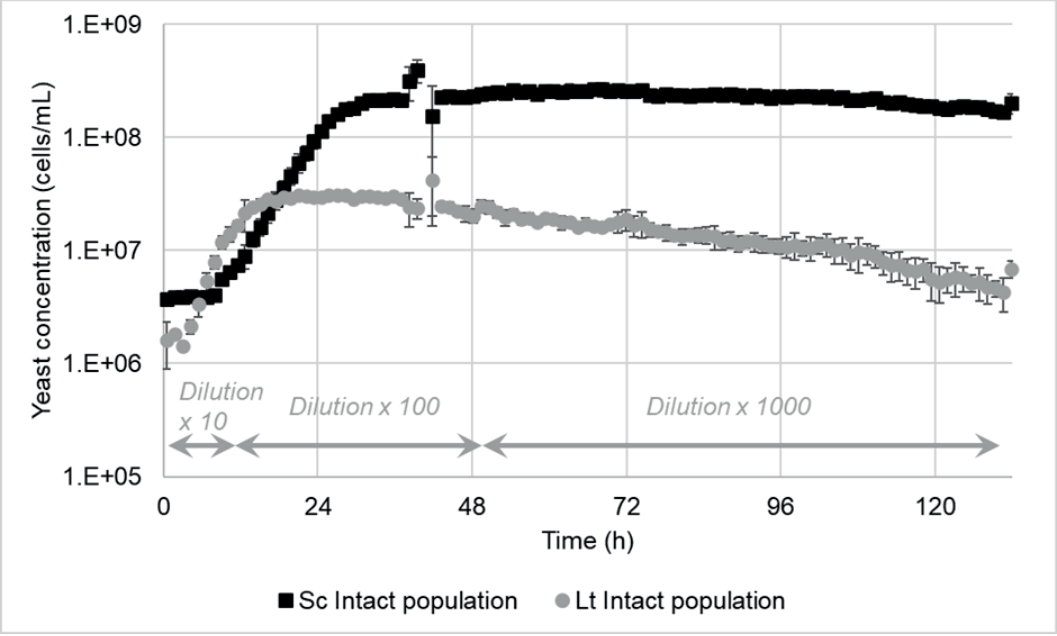


FIGURE 8. Intact population of *Saccharomyces cerevisiae* and of *Lachancea thermotolerans* monitored by automated flow cytometry during mixed fermentation.

Black squares and grey circles represent total and intact yeast concentration respectively. Each square and circle represents the mean of 3 biological replicates. For each replicate, a measurement was taken every 85 min.

4. Microbial monitoring during wine aging

After having studied high-frequency and low duration monitoring, the goal of the final experiment was to explore system stability over long periods of time, especially with less frequent acquisitions. It also allowed us to study yet another matrix - i.e., red wine - and to validate the compatibility of this technology with this matrix.

The dot plots in Figure 9A show the signals obtained during the monitoring of a microbial population in a sample of red wine. The "SG +" gate discriminated the microbial population from the expected background noise of the complex medium,

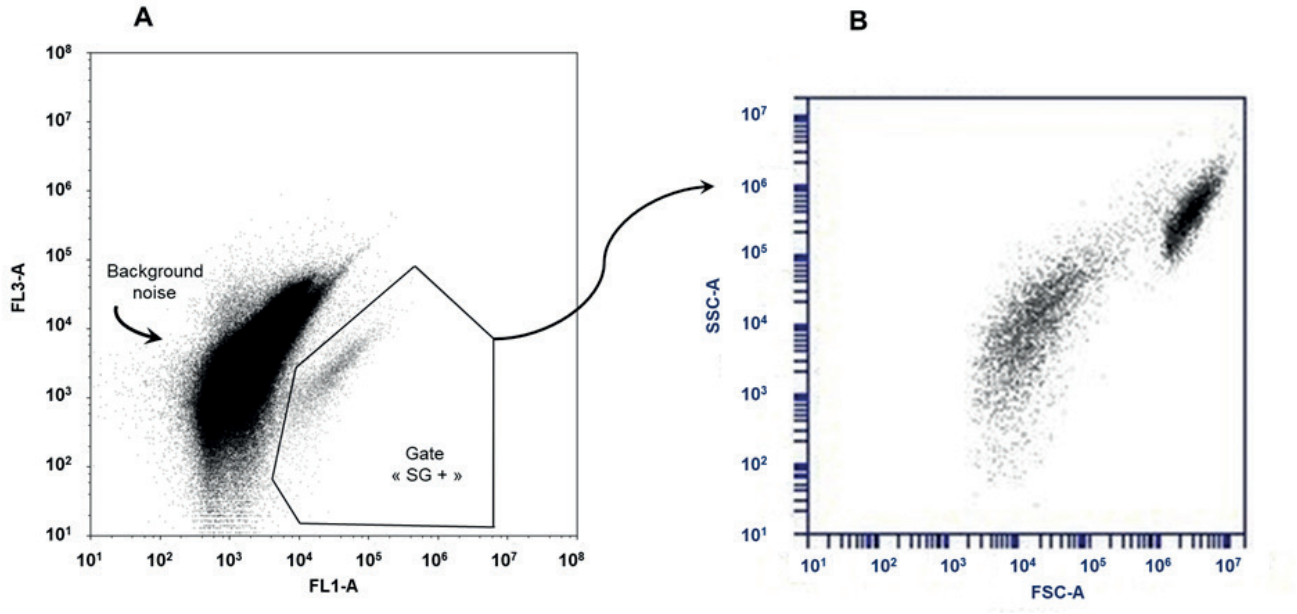


FIGURE 9. Exemplary density plots (t = 256 h) obtained with automated flow cytometry during the monitoring of a microbial population in red wine during aging after staining with SG (A) and size distribution of events from the "SG +" gate.

In Figure 9A, each dot represents the fluorescence intensity of a given particle in the green fluorescence range (x-axis, FL1-A, logarithmic) and the red fluorescence range (y-axis, FL3-A, logarithmic). The "SG +" gate delineates the total microbial population labeled by SG. In Figure 9B, forward scatter is plotted on the x-axis and side scatter on the y-axis, both with logarithmic scales.

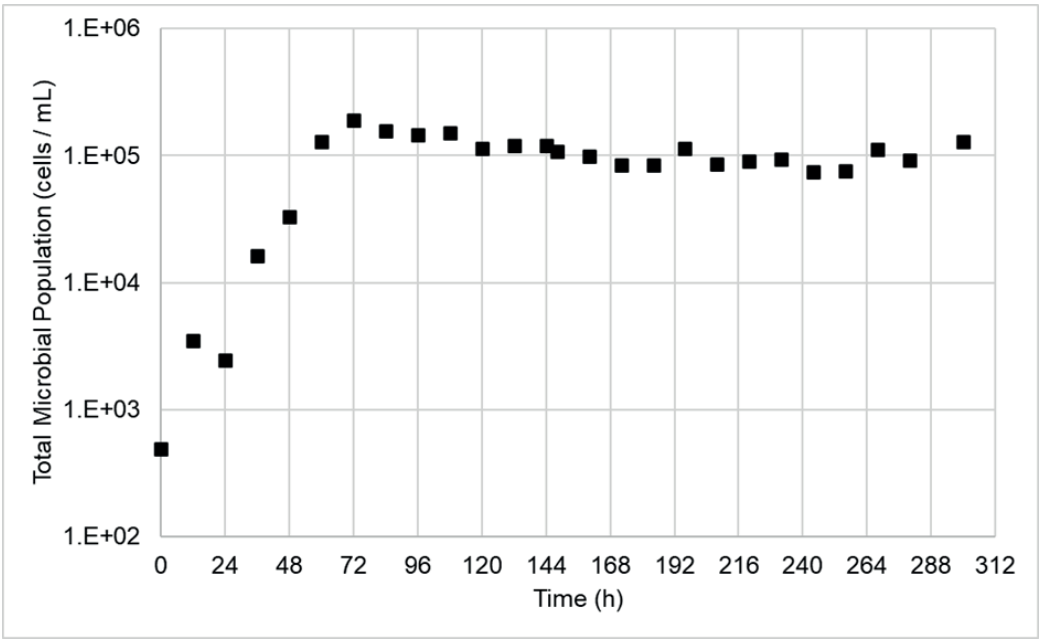


FIGURE 10. Total microbial population (yeast and bacteria) ("SG +" gate of Figure 9) during wine aging, monitored by automated flow cytometry.

A measurement was taken every 12 h.

red wine (autofluorescence of numerous compounds, cellular debris and others).

The microbial population that showed a green fluorescence in the presence of SG consisted of two populations, small particles and bigger particles, which can be associated with the bacterial population and the yeast population respectively, given the FSC and SSC values of the corresponding events (Figure 9B).

Total microbial population (Figure 10) increased during the first 72 h and then remained stable during the following 10 days. Some instabilities in the estimated population were observed at the beginning of the experiment, which could

be explained by the very low level of population, both in absolute terms and relative to the background noise.

DISCUSSION

The overarching goal of this study was to evaluate the potential and feasibility of applying automated flow cytometry to wine-related environments, by carrying out a wide range of increasingly complex case studies, each aiming to explore different technical challenges. Overall, even if flow cytometry is nowadays widely used in wine-related scientific experiments (Bordet *et al.*, 2020; Guzzon and Larcher, 2015; Longin *et al.*, 2017) and has even begun to work its way into more and more cellar laboratories, automated flow cytometry has not yet been applied in these conditions (Heins *et al.*, 2022). It indeed poses a few challenges, like those related to high-sugar medium sampling, dissolved CO_2 management or high growth, but there is a need in this field of research for the facilitation for higher-frequency microbial sampling and analysis, and around-the-clock measurements. The automation of flow cytometry could constitute part of the answer to all of these issues.

As a result of the first experiments (Results, Part 1), which were conducted in simple conditions (presence of one type of microorganism, physiological water matrix), it was possible to validate the protocols for sampling, staining and flow cytometry analysis. An intentional longer than usual rehydration period allowed us to both validate the longer acquisition times and observe a population shift during the experiment on bacteria. The data show that the automated flow cytometry technology can be used to monitor a yeast population for several hours after rehydration, with good repeatability. The double staining method can be used to quantify total and intact yeast concentrations. The experiment on bacteria led to similar conclusions being made regarding method validation, and it also highlighted a decrease in total population while the intact bacterial population remained constant. These results can be explained by a very long monitoring period in comparison to the usual rehydration duration. Rapid consumption of the growth factor and subsequent nutrient scarcity led to a decrease in total bacterial concentration. Moreover a resulting possible autolysis phenomenon (already observed in *O. oeni* (Crouigneau *et al.*, 2000)) can explain the meanwhile relatively stable intact population. This hypothesis can be further confirmed by comparing the density plot of Figure 3 with one of the density plots generated at the end of the experiment (see Supplementary data, Figure S2), and observing the disappearance of the "damaged bacteria" event cloud. Therefore, the validation of the chosen automated flow cytometry method via the analysis of both yeasts and bacteria in simple media - as has been done in other studies (Besmer *et al.*, 2014) - proved the studied system was capable of accurately evaluating microbial populations in "ideal" conditions. These results were essential, as they paved the way to the further experimentation presented in this paper.

The second part of the study (Results, Part 2) explored the possibility of monitoring microbial populations over multiple days, in a more complex medium (water-sugar-wine mix). The results here were of significant importance for multiple reasons. First, they validated that the system can monitor microbial cultures - both total and intact populations - overnight for 72 h without drift, thus showing the efficiency of the automated clean-in-place protocol. Second, the results showed that the studied system is able to automatically monitor yeast populations in a complex medium (containing high concentrations of sugars and ethanol, in the presence of gas bubbles and others). This is quite novel, as it is the first time automated flow cytometry has been applied with success to wine must under fermentation - a medium with high sugar content, and consequently a higher viscosity than the minimal media used in previous experiments involving microbial growth (Baert *et al.*, 2015; Freitas *et al.*, 2013; Kacmar *et al.*, 2004).

In addition, these experiments validated the possibility of monitoring exponential growth using different predilution factors. However, altering the dilution factor (from 10-fold to 100-fold) caused a jump in the calculated concentration. Possible explanations for this are an underestimation of the population concentration before the higher sample dilution, overcrowding of the cytometer's sensors or a poor separation of microorganisms (leading to yeast duets and triplets being measured as a single event). The higher dilution may have resulted in a higher yeast population, because the ratio of cells to background in the SG gate was higher; hence, the multiplication by 100 instead of by 10 will have had a stronger effect on the back-calculated concentration. Moreover, these results confirm that the application of such a system to short-term high-frequency monitoring of yeast populations during a levain preparation is possible, especially for the "Prise de Mousse". This could be useful for future experiments, because such frequent microbial monitoring is very labour intensive and time consuming (even with flow cytometry), thus not always easy to put in place. For this reason, existing studies often favour lower analysis frequency (Benucci *et al.*, 2016; Kemp *et al.*, 2020), and similar future ones could benefit from easier access to higher sampling frequencies.

The third part of the study (Results, Part 3) was built on the aforementioned growth-monitoring capabilities and allowed longer monitoring to be carried out, with higher growth and population levels, than the previous part involving levain preparation. The instability of the results observed at about 40 h quickly resolved without any external intervention, and may have been the result of multiple external factors impacting the analysis system, from a manipulation error when refilling the reagents to a bubble having entered the sampling system, or maybe an isolated agitation problem.

In addition, the aim of this experiment was to evaluate the capacity of alcoholic fermentation monitoring, which had not yet been explored through the prism of automated flow cytometry (especially involving a mixed culture). Indeed, this type of monitoring constitutes a technical challenge: rapid microbial growth during the first few days of fermentation,

then high stability, CO_2 production, transition from a high-sugar to a low-sugar medium, high ethanol media, long duration (especially at 20 °C). This experiment highlighted the possibility for this system to monitor total yeast populations and intact yeast populations - two key variables for properly managing this crucial step of winemaking - over the course of the alcoholic fermentation process. The experiments were conducted using synthetic wine must, which was designed to behave similarly to white wine must (Bely *et al.* 1990); however, the results need to be confirmed in further experiments using red wine must, as the presence of lees and pigments could hinder the analysis.

Furthermore, as with standard flow cytometry and due to the use of a fluorescent mutant, it was possible to obtain a sound differentiation of two different yeast species over time. Being able to monitor several microbial populations simultaneously throughout their growth phase and the subsequent stationary phase is also essential when monitoring mixed cultures in laboratory-scale experiments. Increasing the acquisition frequency is both very valuable and quite challenging for studies on this subject. Use of this technology can also be envisioned to monitor mixed fermentations involving yeast populations distinguishable without fluorescing mutants, like *Starmerella bacillaris* and *S. cerevisiae* (Gobert, 2019).

The overall precision of the results and the fact that the experiment did not encounter any significant technical issues indicate that the system can be used for research experiments involving alcoholic fermentations in the future. Indeed, such experiments could, like those involving levain monitoring, also benefit from the higher frequency of microbial sampling, especially during the exponential growth phase when μ_{max} is often evaluated. Flow cytometry use is already quite developed for such experiments (Bordet *et al.*, 2020; Pérez-Torrado *et al.*, 2017), thus only minimal adjustments to existing methods would be required.

However, a limiting factor would be the permanent agitation required for such monitoring, interfering with the usual static conditions and sedimentation during the later phases of fermentation, and thus modifying the very biological system meant to be observed (Varela *et al.*, 2021). In this regard, automated flow cytometry is indeed no different from standard high-frequency manual sampling, which suffers from the same dilemma. Some ways of indirectly dealing with this would be either to 1) accept the possible differences and check for them via a static experiment conducted in parallel, 2) lower the acquisition frequency and link the agitation to the sampling system to simulate lower frequency manual samplings, or 3) keep the static conditions and only sample the supernatant (Veloso *et al.*, 2020).

The fourth and final part of the study (Results, Part 4) focused on wine aging and tested radically different experimental conditions from the other parts: low population dynamics, longer time gaps between the samples, and a very long monitoring duration. The automated flow cytometry system allowed the global microbial population to be monitored in a red wine for a long time (13 days); no outlier results were obtained, indicating that the sampling, analysis and clean-in-place protocol were efficient, even when carried out over several weeks and at considerably lower sampling frequency. These first results indicate that such a system, with proper setup and method development, could be used in the future to monitor and study wine as a microbial system all along the aging process. It would be especially interesting for monitoring malolactic fermentation or abnormal microbial development, or even specific alteration floras if identifiable by flow cytometry, such as *Brettanomyces bruxellensis* with RNA-FISH probes (Branco *et al.*, 2020).

However, the experiment as it stands has several shortcomings. The first one is the very high background noise, which was expected given that the analysed sample was unfiltered red wine that was barely diluted, thus containing debris and particles of the same size as microorganisms (Salma *et al.*, 2013). SYBR Green I staining helped alleviate this problem and identify microorganisms among the other particles, but any saturation of the cytometer detectors and aggregation between microorganisms and particles must still be considered. In addition, as in the case of non-automated flow cytometry, particular attention should be paid to the interference with natural fluorescence of anthocyanin adsorbed on yeasts in red wine (Echeverrigaray *et al.,* 2020). Second, the experiment was only run for 13 days, but in a cellar, a wine monitoring programme would in theory need to run for many weeks, even months. In this interval, some grime build-up - not observed within the time frame of this study - could occur over time, and the system would then need some supplementary maintenance outside of the clean-in-place protocol used here. Third, the experiment lacks repetitions; therefore, these preliminary results will have to be confirmed by further studies. Nevertheless, the results open the door to potential applications of automated flow cytometry to future monitoring of laboratory-scale malolactic fermentation or wine aging.

Overall, even if some adjustments and method refining are required, these results hint at a high diversity of potential applications for automated flow cytometry; for example, monitoring microbial starter pre-adaptation, alcoholic and malolactic fermentations or wine aging. Such uses in both laboratory and cellar conditions could also be coupled with other existing on-line analysis techniques, such as FTIR for analysing multiple physico-chemical characteristics of the wine (Veale *et al.*, 2007; Veloso *et al.*, 2020), or thermal conductivity for carbon dioxide monitoring (Descoins *et al.*, 2006), thus fully automatising most of the tedious parts of the monitoring process. Data analysis automation, such as automatic gating, could also be a useful addition to this technique, as mentioned by Heins *et al.* in their recent review (Heins *et al.*, 2022).

CONCLUSIONS

The present study shows the technical potential of using automated flow cytometry in laboratory-scale winemaking experiments. Using this system, it is possible to monitor the

bacteria and yeasts involved in wine production in a complex sugar- and ethanol-rich medium over a long period of time (up to 2 weeks). A variety of applications of the system, which are common in this field, were tested. The results show the applicability of the studied automated system for both high-frequency and around-the-clock sampling. Overall, this study constitutes a first step towards the automation of flow cytometry analysis in both wine research and the wine industry, which could in the near future constitute the next tool of interest for both microbiology studies and on-line quality control.

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