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Utilization of the AIRMIXING M.I.™ System in Producing Red Wine without Added Sulphites

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Abstract: Sulphur dioxide (SO_2) is usually used in winemaking due to its effectiveness as an antimicrobial and antioxidant agent. However, there is growing interest in finding alternatives to SO_2 because of its adverse effects on human health. Therefore, in this work, a low-SO_2-producing *Saccharomyces cerevisiae* strain was selected and the AIR-MIXINGTM M.I^{TM} (A.M.) system, capable of determining a high extraction of polyphenols, was used to produce a red wine without added sulphites. A tank managed with the traditional pumping-over technique was used as a comparison. Microbiological and chemical monitoring of both fermentations performed by plate counts and HPLC analyses, respectively, did not indicate significant differences as regards the yeast growth kinetics and the degradation of the sugars, while it highlighted a faster extraction kinetics of colour and total polyphenols in the fermentation carried out with the A.M. system. Both experimental wines showed a total SO_2 content <10 mg/L, but in the wine produced with A.M., a higher content of the polymeric forms of anthocyanins and non-anthocyanin phenols was found in favour of a higher stability of the wine achieved in a shorter time than the control. Furthermore, a higher concentration of reduced glutathione, a compound well-known for its antioxidant activity, occurred in wine obtained with the A.M. system. In conclusion, the use of low-SO_2-producing yeasts in combination with the A.M. system could be a suitable approach to produce wines without sulphites added.

Keywords: sulphur dioxide; sulphites added; red wine; polyphenol content; low-SO_2-producing *S. cerevisiae* strains

Citation: Guerrini, S.; Barbato, D.; Mangani, S.; Mari, E.; Buscioni, G.; Ganucci, D.; Galli, V.; Granchi, L. Utilization of the AIRMIXING M.I.™ System in Producing Red Wine without Added Sulphites. *Fermentation* **2023**, *9*, 812. https://doi.org/10.3390/fermentation9090812

Academic Editor: Alice Vilela

Received: 28 July 2023
Revised: 22 August 2023
Accepted: 4 September 2023
Published: 6 September 2023

1. Introduction

Sulphur dioxide (SO_2) is mainly used in wine as an antiseptic agent against microorganisms, as an antioxidant agent, and as an enzyme inhibitor [1]. In grape must, SO_2 reduces the oxidation and the total microbial load favouring the selection of *Saccharomyces cerevisiae*, thus reducing the risk of having non-optimal fermentation processes. After malolactic fermentation, the use of SO_2 is aimed at eliminating spoilage bacteria, such as lactic acid and acetic acid bacteria, and yeasts such as *Brettanomyces/Dekkera* responsible for sensorial defects related to the production of volatile phenols [2]. Sulphites also offer protection from the oxygen that dissolves in the grape must and in the wine from the air during the operations of vinification. Oxidation reactions can positively or negatively affect the quality of the wine based on its composition and exposure to oxygen during winemaking. Sulphites such as molecular SO_2, bisulphite ions HSO_3^-, and/or combined SO_2 can interfere with this process. For example, SO_2 is effective in preventing the degradation of numerous molecules involved in the aromas and colour of the wine, while the form HSO_3^- can inhibit oxidative enzymes in musts, as well as preventing oxidative browning

in white wines [3]. SO_2 can also react with quinones, reducing them to phenols [4,5], and with aldehydes such as acetaldehyde, with consequent positive effects in reducing the perception of typical defects of oxidation [5]. SO_2 and hydrogen sulphide (H_2S) are naturally produced also by yeasts, during the sulphur assimilation pathway, at a different level depending on the strain. Therefore, the uncontrolled use of sulphites as preservatives could confer the wine-negative rotten egg aroma, usually associated with H_2S and thiol compounds [6–9], as well as inhibiting malolactic fermentation and representing a risk to human health. SO_2-derived compounds negatively affect human health, causing symptoms such as urticaria, angioedema, dermatitis, diarrhoea, abdominal pain, bronchoconstriction, and anaphylaxis in sensitive individuals. The toxicity and the allergenic potential of SO_2 are also of concern, since this compound is widely used as a preservative in a lot of foods and accumulates in human organisms [6–9]. Taking these aspects into consideration, the World Health Organization (WHO) has established an acceptable daily intake (ADI), for SO_2 of 0.7 mg/kg of body weight, and the European Union legislation requires the indication "contains sulphites" to be indicated on the label if the total SO_2 contained in wine is higher than 10 mg/L (Directive 2003/89/CE). For all these reasons, sulphur compounds represent one of the most important parameters determining the acceptability for wine marketing [7]. Therefore, the general trend is to reduce the use of SO_2 in winemaking processes. Recently, various authors have proposed new biotechnological approaches to obtain a significant reduction in sulphites in wines, such as the use of alternative additives and innovative physical methods [1,2,10]. Only some alternative additives have been already authorized in winemaking, while other systems have only been tested at the experimental level [2]. However, based on current knowledge, none of these methods have proven to be able to completely replace SO_2. Although the guidelines reported in the OIV-OENO 631-2020 resolution [11] of the International Organization of Vine and Wine (OIV) can help wine producers to limit the presence of SO_2 in wine, further studies are needed to define appropriate management winemaking practices without the use of this preservative. Therefore, this study aimed to evaluate the possibility of producing wines without added sulphites by using low SO_2-producing *S. cerevisiae* strains to conduct the alcoholic fermentation and the Air-Mixing M.I.™ system, an innovative technique to manage red grape maceration in association with the macro-oxygenation that enhances antioxidant properties of the wine. Indeed, the Air-Mixing M.I.™ system, according to a recent study [12], seems to produce wines characterized by a greater extraction of polyphenols than the wine obtained with the traditional pump-over system. This higher content of antioxidant compounds makes the Air-Mixing system a suitable practice to replace in part the SO_2, and thus useful for producing wines without sulphites added. Therefore, SO_2-free vinifications were carried out with the Air-Mixing M.I.™ system, and the traditional method of pumping over and the wines obtained were compared by assessing physicochemical and microbiological parameters during the alcoholic fermentation and after three months of ageing, including, at this stage, the sensory analysis.

2. Materials and Methods

2.1. Laboratory-Scale Fermentation in Synthetic and Natural Grape Must to Select a Low-SO_2-Producing Saccharomyces cerevisiae Strain

Ten commercial *S. cerevisiae* strains (VIW® Cleaver, VIW® Fruity, Zymaflore RB4, Zymaflore FX19, Zymaflore X16, EnartisFerm SC, EC1118, CRU31, Anchor Vin13, ICV Opale YSEO 2.0) were chosen as in their technical schedule, were indicated as low or medium SO_2 producer. To confirm their properties and chose the lower SO_2 producer, the strains were inoculated at 2×10^6 cell/mL as axenic cultures in 250 mL Erlenmeyer flasks, sealed with a Müller valve filled with sulphuric acid and containing 160 mL of synthetic must [13]. The most promising were successively tested on grape must (glucose, 141 g/L; fructose, 144 g/L; yeast assimilable nitrogen, 300 mg/L; total SO_2, <10 mg/L; pH = 3.32). The fermentations were carried out in duplicate at 25 °C and monitored daily, recording the weight loss until the measure was constant for three consecutive determinations. The

data of each assayed strain were interpolated with the Gompertz function to calculate the specific fermentation rate μ (h^{-1}). Substrate and main product metabolisms of the starter strains were detected at the end of the alcoholic fermentation as reported below.

2.2. Cellar Fermentations Realised with Air-Mixing M.I.™ and with the Traditional System

Grapes of Sangiovese, Merlot, and Colorino varieties were collected during the 2022 harvest, and the obtained grape juice was used for experimental trials in the cellar (Magliano, Tuscany, Italy). The grape juice was divided into two stainless steel tanks (50 hL of volume) both equipped with a cooling jacket for temperature control, set at the same temperature. One of the tanks was also equipped with AIRMIXING M.I.™ (Parsec s.r.l., Sesto Fiorentino, Italy). As reported by Pettinelli et al. [12], the AIRMIXING technique consisted of the injection of air jets from three nozzles. These nozzles were set up to inject the air jets, creating a movement of the fermenting must that was able to keep the cap soft and the mass temperature more uniform. For the control trial, the vinification protocol used in the cellar for red wine, involving twenty minutes of pumping over the first three days of fermentation, was applied. The chemical composition of the grape juice was as follows: glucose, 104 g/L; fructose, 118 g/L; malic acid, 0.8 g/L; total nitrogen, 190 mg/L; pH 3.34. The two tanks were inoculated with the same low-SO_2-producing *S. cerevisiae* strain (ICV Opale YSEO 2.0) at 2×10^6 CFU/mL. The fermentations were chemically and microbiologically monitored as reported below. At the end of the alcoholic fermentation, the wines were racked off and analysed to quantify amino acids, phenols, biogenic amines, and glutathione contents. The same microbiological and chemical analyses were conducted after three months of ageing. All the analytical methods used are reported below.

2.3. Microbiological Analysis

2.3.1. Microorganism Quantification

Microbial analyses were carried out as reported by Guerrini et al., 2021 [14]. Yeasts were quantified on WL Nutrient Agar medium (Oxoid Ltd., Basingstoke, Hampshire, UK) containing sodium propionate (VWR International Srl, Milan, Italy) (2 g/L) and streptomycin (VWR International Srl, Milan, Italy) (30 mg/L) to inhibit mould and bacteria growth, respectively. The plates were incubated for 48 h at 30 °C in aerobic conditions. Lactic acid bacteria were quantified on MRS Agar medium (Oxoid Ltd., Basingstoke, Hampshire, UK) incubated for five days at 30 °C in anaerobic conditions, acetic acid bacteria were quantified on a Lafon-Lafourcade medium (glucose, 10 g/L; yeast extract, 5 g/L; peptone, 5 g/L; tomato juice broth, 2 g/L; agar, 20 g/L; pH 5.00). Pimaricin at 50 mg/L was added to the MRS and Lafon-Lafourcade media as a yeast inhibitor. *Brettanomyces bruxellensis* was quantified on the DBDM medium (yeast extract, 2% (*w/v*) peptone, 2% (*w/v*) glucose).

2.3.2. Microorganism Identification

Yeast isolates were identified by amplification of the 5.8S rRNA gene and of the two ribosomal internal transcribed spacers, using the primer pair ITS1/ITS4 followed by restriction with HaeIII (Life Technologies Italia, Monza, Italy), as described by Granchi et al. [15]. To confirm the dominance of the yeast-inoculated strains, isolates were characterized at strain level by inter-δ PCR typing with δ12/δ21 primer pair (Thermo Fisher Diagnostics S.p.A., Rodano, Milan; Italy) as reported by Legras and Karst [16]. *Oenococcus oeni* isolates were identified as reported by Zapparoli et al. [17].

2.4. Chemical Analysis

2.4.1. Substrates and Products of the Main Metabolism

Glucose, fructose, ethanol, glycerol, 2,3-butanediol, and acetic, lactic, and succinic acid contents in must and wine were determined by HPLC, according to Guerrini et al. [18], utilizing a Rezex ROA-Organic Acid H+ (8%) column (8-μm particle, 300 × 7.8 mm; Phenomenex, Torrance, CA, USA) and a ProStar 210 chromatograph equipped with a

DAD at 210 nm and a refractive index detector, in series (Varian Inc., Palo Alto, CA, USA). The malic acid concentration was determined enzymatically through an automatic multi-parametric analyser (Hyperlab, Steroglass, San Martino, Italy).

2.4.2. Glutathione

Reduced and oxidized glutathione were determined as described by Guerrini et al. [19].

2.4.3. Total Phenols

Total phenol index (TPI) was measured as absorbance at 280 nm, according to Mangani et al. [20].

2.4.4. Amino Acids

Amino acids were quantified as dansyl-derivative as described by Tuberoso et al. [21], using heptylamine as an internal standard. Determination was carried out with an HPLC- UV/FLD Jasco series 4000 (Jasco, Japan Spectroscopic co, Hachioji city, Japan) equipped with a pump PU-4180, an autosampler AS-4050, a photodiode array detector MD-4010, a fluorescence detector FP-4025, and a column oven CO4060 equipped with a 150 mm × 4.6 mm × 5 µm Gemini® C18 column (Phenomenex Inc, Torrance CA, USA) protected by a C18 SecurityGuard® cartridge.

2.4.5. Biogenic Amines

Biogenic amines (agmatine, ethanolamine, phenylethylamine, cadaverine, histamine, tyramine, spermine, and spermidine) were quantified as dansyl-derivatives as described by Tuberoso et al. [21], using heptylamine as internal standard. The reaction mixture consisted of 100 µL wine, 100 µL dansyl chloride solution (derivatization agent) and 0.2 M $Na_2B_4O_7 \cdot 10H_2O$ (pH 9.3) solution up to a final volume of 1000 µL. The mixture was incubated for 30 min at 40 °C and filtered at 0.45 µm before injection. Determination was carried out with an HPLC-UV/FLD Jasco series 4000 (Jasco, Japan Spectroscopic co, Hachioji city, Japan) equipped with a pump PU-4180, an autosampler AS-4050, a photodiode array detector MD-4010, a fluorescence detector FP-4025, and a column oven CO4060 equipped with a 150 mm × 4.6 mm x 5 µm Gemini® C18 column (Phenomenex Inc, Torrance, CA, USA) protected by a C18 SecurityGuard® cartridge. Quantification was performed using calibration curves obtained according to the internal standard method, which correlates the analyte/IS peak area ratio with concentration. Biogenic amine standards, heptylamine, and dansyl chloride were from Merck (Merck Life Science, Milano, Italy). All solvents were of HPLC quality, and all chemicals were of analytical grade (>99%); water was of Milli-Q quality (Millipore, Billerica, MA, USA).

2.4.6. Phenols

For the determination of hydroxybenzoic and hydroxycinnamic acids, flavonols, flavan-3-ols, stilbenes, and phenolic alcohols, wines were filtered (0.45 µm) and injected into the HPLC- UV/FLD Jasco series 4000 (Jasco, Japan Spectroscopic co, Hachioji city, Japan) equipped with a pump PU-4180, an autosampler AS-4050, a photodiode array detector MD-4010, and a column oven CO4060 and a reversed-phase column NovaPak C18 (4-µm particle, 300 × 3.9 mm; Waters, Milford, MA, USA), thermostated at 25 °C. The mobile phase was (A) 2% (*v/v*) acetic acid in water and (B) acetonitrile; the gradient profile was 0–40 min, 1–20% B; 40–45 min, 20–50% B; and 45–55 min, 50–95% B, followed by washing with acetonitrile and re-equilibration of the column from 65 to 85 min; the flow rate was 0.9 mL/min from the beginning to 35 min and 1.0 mL/min from this point to the end. Phenolic compounds were detected by scanning from 210 to 600 nm. Hydroxybenzoic acids were quantified at 280 nm using gallic, protocatechuic, vanillic, and syringic acids as standards (Merck Life Science, Milano, Italy); methylgallate and ethylgallate were expressed as gallic acid equivalents. Hydroxycinnamic acids were quantified at 280 nm using caffeic, *trans*-p-coumaric and ferulic acids as standards (Merck Life Science, Milano,

Italy); fertaric acid was expressed as ferulic acid equivalents, trans-caftaric acid as caffeic acid equivalents, and *trans*-p-coumaric and *cis*-p-coumaric acids as *trans*-p-coumaric acid equivalents. Stilbenes were quantified at 280 nm using trans-resveratrol and cis resveratrol as standards (Merck Life Science, Milano, Italy); *trans*-piceid and *cis*-piceid were expressed as trans-resveratrol and cis-resveratrol equivalents. Flavan-3-ols were quantified at 280 nm using catechin and epicatechin as standards (Merck Life Science, Milano, Italy); epicatechin-3-O-gallate was expressed as epicatechin equivalents. Flavonols were quantified at 360 nm using quercetin, myricetin, kaempferol, quercetin-3-*O*-glucoside, quercetin-3-*O*-glucuronide, quercetin-3-*O*-galactoside, kaempferol-3-*O*-glucoside (Merck Life Science, Milano, Italy), myricetin-3-*O*-glucoside, and myricetin-3-*O*-galactoside (Extrasynthese, Cedex, France) as standards. Phenolic alcohols were quantified at 280 nm using tyrosol and tryptophol as standards (Merck Life Science, Milano, Italy); hydroxytyrosol was expressed as tyrosol equivalents. Volatile phenols (4-vinyl-phenol, 4-vinylguaiacol, 4-ethylphenol and 4-ethylguaiacol) were determined by HPLC- UV/FLD Jasco series 4000 (Jasco, Japan Spectroscopic co, Hachioji city, Japan) equipped with a pump PU-4180, an autosampler AS-4050, a photodiode array detector MD-4010 at 280 nm, a fluorescence detector FP-4025 (λexc 260/λems 305), a column oven CO4060, a reversed-phase column Kinetex (5-µm particle, 150 × 4.6 mm; Phenomenex, Torrance, CA, USA), and a thermostated at 25 °C. The mobile phase was (A) 0.1% (*v/v*) phosphoric acid in water and (B) acetonitrile; the gradient profile was 0–25 min, 10–90% B; 25–30 min, 90–10% B; followed by 15 min re-equilibration of the column; the flow rate was 1.0 mL/min.

2.5. Sensory Analysis

The sensory analysis of the wines produced was performed according to the methods reported by Resolution OIV/CONCOURS 332A–2009 [22].

2.6. Statistical Analysis

Analytical determinations, performed in duplicate, were elaborated according to the *t*-Test or nonparametric ANOVA followed by Tukey's test. Differences were reported at a significance level of $p < 0.05$ or $p < 0.01$. All of the statistical analyses were performed by Statistica 7.0 software package (Stasoft GmbH, Hamburg, Germany).

3. Results

3.1. Laboratory-Scale Fermentation in Synthetic and Natural Grape Must to Select a Low-SO_2-Producing Saccharomyces cerevisiae Strain

One of the first steps in producing a wine with a sulphite content lower than 10 g/L (the value required to market a wine without sulphites added) is to use a low-SO_2-producing *S. cerevisiae* strain to carry out the alcoholic fermentation. Therefore, ten commercial *S. cerevisiae* strains marketed for their capacity to produce low or medium SO_2 amounts were tested through 160 mL fermentations on synthetic must. The aim was to evaluate their effective capability to produce low SO_2 quantities and choose the lower-producing strain. Each strain was inoculated, as axenic culture, at a concentration of 2×10^6 CFU/mL. After ten days at 25 °C, the sugar consumption was completed with low acetic acid production (<0.3 g/L) for all the tested strains. All the strains also demonstrated high fermentative vigour (CO_2 produced in 48 h higher than 6 g/100 mL) and comparable fermentative performance, estimated as specific fermentation rates with the Gompertz model. However, differences were found in SO_2 production which ranged between 17 and 40 mg/L; the lower-producing strains were Zymaflore RB4, EnartisFerm SC, Anchor Vin 13, and ICV Opale YSEO 2.0 (Figure 1).

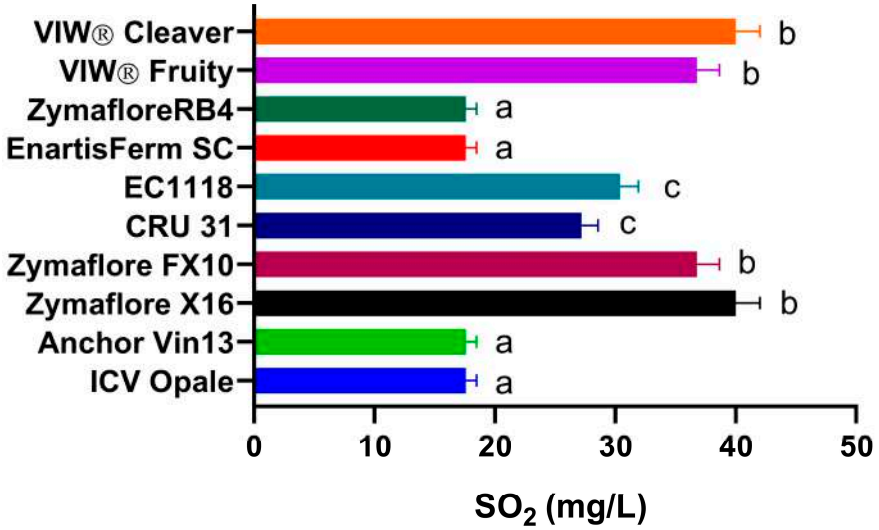


Figure 1. Total SO_2 concentrations (mg/L) produced by 10 commercial starter strains at the end of the fermentations in synthetic must. Different letters indicate significant differences among samples (ANOVA, Tukey test at $p < 0.01$).

These strains were further tested on natural must, obtained from grapes supplied by the cellar where the subsequent experimental steps were carried out. Figure 2 shows the fermentative kinetics expressed as weight loss over time due to the release of CO_2. When CO_2 production became stable, yeast isolates from various trials were analysed by inter-delta PCR, which confirmed the complete dominance (100%) of the inoculated starter strain in each fermentation. The results of the fermentation profile pointed out significant differences among the strains, with the strain ICV Opale YSEO 2.0 being the most performant, as shown by the maximum fermentation rate estimated with the Gompertz model (Table 1). The results of chemical analyses of the experimental wines after 15 days from the yeast strain inoculation demonstrated that the sugar consumption was completed only for the strains ICV Opale YSEO 2.0 and Anchor Vin 13; therefore, the other strains were discarded.

Both yeast strains that completed the sugar degradation produced high acetic acid concentrations, probably because of the high initial sugar concentration (270 g/L) present in the must, which determined possible osmotic stress. Since the ICV Opale YSEO 2.0 strain produced significantly lower SO_2 than the Anchor Vin 13 strain and showed the highest fermentation rate, it was chosen to carry out the alcoholic fermentations realized with the Air-Mixing M.I.™ and with the traditional systems in the cellar.

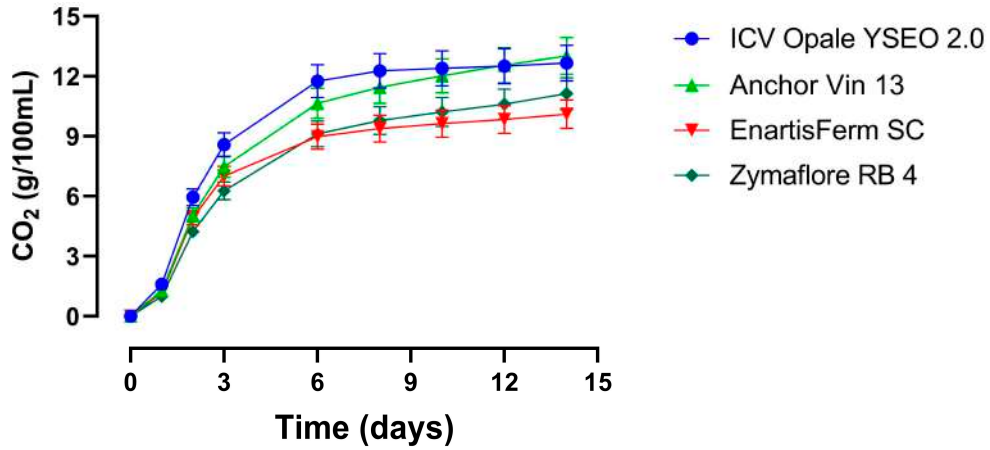


Figure 2. Fermentation kinetics in grape must inoculated with 4 *S. cerevisiae* commercial strains.

Table 1. Chemical analyses of the experimental wines obtained with 4 *S. cerevisiae* commercial starters and fermentative performance of the strains in terms of specific fermentation rate (μ-max) estimated with the Gompertz model. Different letters in the same row indicate statistically significant differences (ANOVA and Tukey's test, $p < 0.05$).

	ICV Opale YSEO		EnartisFerm SC		Zymaflore RB4		Anchor Vin13	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Glucose (g/L)	0.35 a	0.01	5.72 b	0.14	2.41 c	0.21	0.39 a	0.04
Fructose (g/L)	1.55 a	0.08	45.32 b	0.21	31.80 c	0.14	3.87 d	0.19
Ethanol (%*v*/*v*)	15.56 a	0.02	12.46 c	0.04	13.47 b	0.14	15.40 a	0.20
Glycerol (g/L)	7.48 a	0.07	7.88 b	0.01	7.13 c	0.03	6.83 c	0.13
Lactic acid (g/L)	0.17	0.05	0.20	0.03	0.14	0.04	0.12	0.01
Acetic acid (g/L)	0.75 a	0.03	0.35 d	0.01	0.63 bc	0.03	0.60 c	0.04
Total SO_2 (mg/L)	22.80 a	0.90	32.40 b	1.30	32.40 b	1.30	35.60 b	1.42
Fermentation rate (h^{-1})	3.82 a	0.27	2.66 ac	0.19	3.27 b	0.23	2.29 bc	0.16

3.2. Comparison between the Fermentation Dynamics Obtained with Air-Mixing M.I.™ or with the Traditional System

Grape must was distributed into two 50 hL stainless-steel tanks and inoculated with the ICV Opale YSEO 2.0 strain at 2×10^6 CFU/mL. At the inoculation time, the grape juice contained 1.8×10^6 CFU/mL of non-Saccharomyces yeasts and concentrations of lactic acid and acetic acid bacteria of 1.8×10^3 and 2.2×10^4 CFU/mL, respectively. One tank was managed with the Air-Mixing M.I.™, while the other one with a daily 20 min pumping-over during the first three days of alcoholic fermentation (traditional system). The Air-Mixing M.I.™ technique consisted of the injection of air jets from three nozzles connected through a pipe and laterally placed inside the tank in the lower part. These nozzles were timed to inject the air jets sequentially, hindering the cap formation and favouring the uniform heat distribution into the tank. The frequencies of the air injections, the furnished oxygenation and the temperature monitoring are reported in Figure 3. Although the temperature had been set between 25 and 29 °C, the temperature monitored by the two probes, placed at two different heights of the wine vessel, showed values that reached 36 °C. The same temperature peak at 36 °C was observed in the traditionally managed wine vessel.

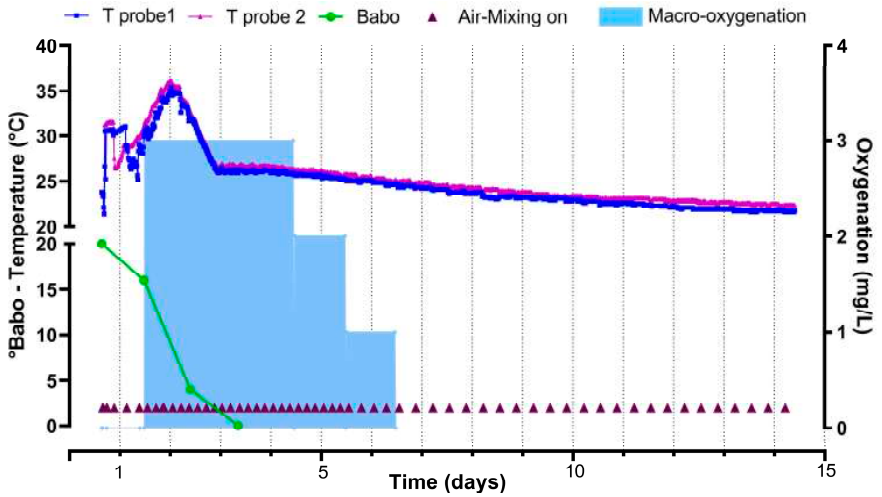
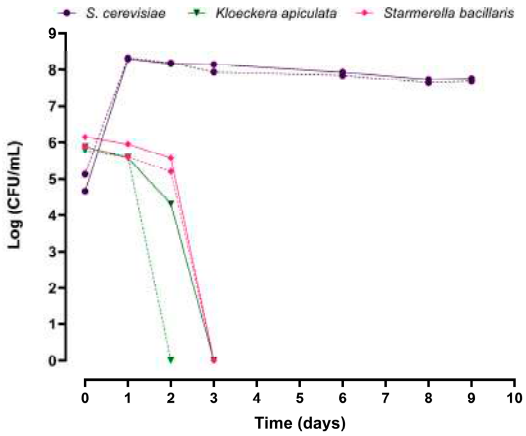


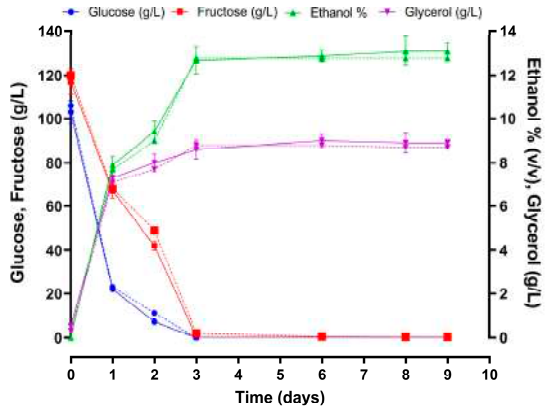
Figure 3. Evolution of tank temperature (°C) and sugar content (°Babo) during the winemaking process managed by the Air-Mixing system and macro-oxygenation (the triangles indicate when Air-Mixing turns on).

This temperature increase determined in both trials a high growth rate of the *S. cerevisiae* population, which quickly reached cell densities of 10^8 CFU/mL 36 h after the yeast inoculum, and a decrease in non-*Saccharomyces* yeasts below the detection limit within the

first three days of fermentation (Figure 4A). In both tanks, the sugar degradation (which was completed in a few days) and the glycerol and ethanol production did not display any significant difference (Figure 4B). Furthermore, both trials showed a negligible presence of acid acetic and lactic acid bacteria during alcoholic fermentation, while at the end *Oenococcus oeni* developed (Figure 4C) and was able to complete malolactic fermentation in the following 15 days in both vinifications. At the end of the fermentations, molecular analysis of the yeast populations confirmed the dominance of the inoculated starter strain ICV Opale ISEO 2.0.



(**A**)



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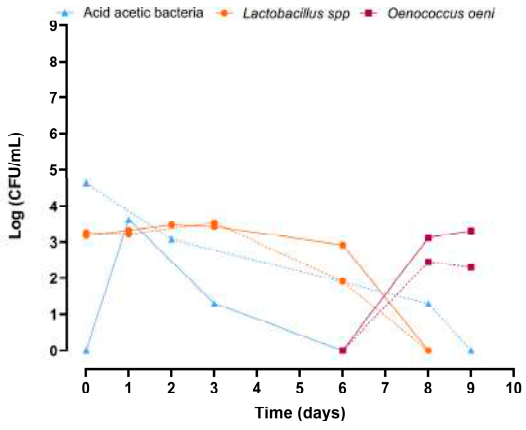

(**C**)

Figure 4. Microbial and chemical evolution of the alcoholic fermentations managed by the Air-Mixing system (continuous line) and traditional system (dashed line). (**A**): yeast concentrations; (**B**): sugar, glycerol, and ethanol production; (**C**): acid acetic and lactic acid bacteria concentrations.

During the alcoholic fermentation, total polyphenol content and colour intensity were also determined in both trials. Results pointed out that the polyphenol extraction was higher in Air-Mixing M.I.™ than in the traditional system (Figure 5). This difference may be attributed to the different management systems used, as the ethanol time course was the same in both fermentations (Figure 4B); therefore, the polyphenol extraction from grape skins was not affected by this metabolite. In any case, at the end of the alcoholic fermentation, the colour intensity was not significantly different in the racked-off wines obtained with the two vinification systems (Figure 5).

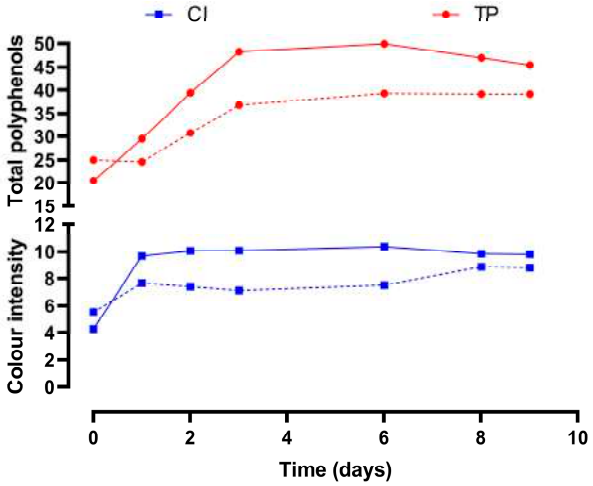

Figure 5. Evolution of total polyphenols (TP) and colour intensity (CI) during alcoholic fermentations managed by the Air-Mixing M.I.™ system (continuous line) and traditional system (dashed line).

3.3. Chemical and Microbiological Characteristics of the Wines at the Racking and after Three Months of Ageing

Fifteen days after the end of alcoholic fermentation, the two wines were racked off in stainless-steel vessels and chemical and microbiological analyses were carried out. The two wines did not show significant differences in the main chemical parameters, and the total and free sulphur dioxide resulted in being below the detection limit in both vinifications (Table 2). Nevertheless, acetic acid was 0.2 g/L higher in wine produced with the Air-Mixing system, probably because of a slightly longer persistence, during alcoholic fermentation, of Kloeckera apiculata, a yeast known for its ability to produce acetic acid. On the contrary, *S. cerevisiae* and O. oeni showed statistically significant differences in cell concentrations. In particular, the *S. cerevisiae* concentrations in Air-Mixing M.I.™ vinification resulted in being two orders of magnitude lower than in the traditional system (Table 2). Moreover, despite the identical fermentation trends of the two trials, almost all the amino acids were present in higher concentrations in the wine obtained with the Air-Mixing M.I.™ system compared to the traditional system (Table 3), probably due to greater yeast autolysis in the wine obtained with the first system. A higher content of free amino acids also corresponded to a greater concentration of biogenic amines after three months of wine ageing, as shown in Table 4. Most of the biogenic amines were significantly higher in the wine obtained with the Air-Mixing M.I.™ system than in that obtained with the traditional system. Some amino acids such as arginine, tyrosine, histidine, and lysine, which were present in higher concentrations in the wine obtained with the Air-Mixing M.I.™ system, are precursors of these toxic substances attributable to the decarboxylase activity of indigenous lactic acid bacteria that carried out the malolactic fermentation.

Table 2. Chemical and microbiological parameters of the wines obtained with Air-Mixing M.I.™ or traditional system after the raking. Different letters in the same row indicate statistically significant differences (*t*-Test, $p < 0.05$).

	Air-Mixing M.I.™ System		**Traditional System**	
	Mean	**SD**	**Mean**	**SD**
Glucose (g/L)	<0.10	-	0.10	-
Fructose (g/L)	0.25	0.01	0.30	0.02
Ethanol (%*v*/*v*)	13.0	0.4	12.9	0.3
Glycerol (g/L)	8.60	0.43	8.50	0.34
Malic acid (g/L)	<0.01	-	<0.01	-
Lactic acid (g/L)	0.66	0.03	0.65	0.04
Acetic acid (g/L)	0.51	0.03	0.29	0.01
Total SO_2 (mg/L)	<10.0	-	<10.0	-
Free SO_2 (mg/L)	<6.0	-	<6.0	-
S. cerevisiae (CFU/mL)	5.50×10^2 a	0.35×10^2	2.55×10^5 b	0.13×10^5
O. oeni (CFU/mL)	2.00×10^3 a	0.18×10^3	2.00×10^2 b	0.08×10^2
Acetic Acid Bacteria	<10		<10	
Brettanomyces spp. (CFU/mL)	<10	-	<10	-

Table 3. Aminoacidic composition of the wines obtained with Air-Mixing M.I.™ or traditional system after raking. Different letters in the same row indicate statistically significant differences (*t*-Test, $p < 0.05$).

	Air-Mixing M.I.™ System		**Traditional System**	
(mg/L)	**Mean**	**SD**	**Mean**	**SD**
Arginine	6.2 a	0.3	6.5 b	0.3
Asparagine	24 a	1.2	17.2 b	0.9
Glutamine	7.3 a	0.4	3.5 b	0.2
Serine	6.8 a	0.3	3.3 b	0.2
Aspartate and glutamate	31.1 a	1.6	16.7 b	0.8
Threonine	5.7 a	0.3	3.5 b	0.2
Glycine	15.4 a	0.8	5.6 b	0.3
Alanine	27.4 a	1.4	9.6 b	0.5
Tyrosine	17.2 a	0.9	5.4 b	0.3
Proline	1693 a	84.7	1609 b	80.4
Methionine	2.5 a	0.1	0.9 b	0.1
Valine	7.0	0.4	4	0.2
Phenylalanine	7.1	0.4	5.1	0.3
Tryptophan	4.7	0.3	2.8	0.1
Isoleucine + leucine	14.7 a	0.7	10.3 b	0.5
Cysteine	25.3 a	1.3	16.9 b	0.8
Histidine	13.3 a	0.7	5.2 b	0.3
Lysine	19.9 a	1.0	14.4 b	0.7

Since it is well known that *S. cerevisiae* during alcoholic fermentation may produce different amounts of reduced glutathione (GSH), which is a natural antioxidant agent effective in the prevention of phenol oxidation in must and wine [23], the glutathione amounts occurring in the two experimental wines were assessed (Figure 6). The concentration of GSH was higher in the wine produced with the Air-Mixing M.I.™ system than in the wine obtained according to the traditional method, both at racking and after three months of ageing. Nevertheless, the reduced glutathione content decreased during ageing in favour of the oxidized form in both wines at almost the same percentage decrease (about 40%).

Table 4. Biogenic amine concentration after three months of ageing in the wines obtained with Air-Mixing M.I.™ or traditional system at raking. Different letters in the same row indicate statistically significant differences (*t*-Test, $p < 0.05$).

	Air-Mixing M.I.™ System		Traditional System	
(mg/L)	**Mean**	**SD**	**Mean**	**SD**
Agmatine	3.8	0.19	3.8	0.19
Ethanolamine	21.4 a	1.1	19.5 b	0.975
Phenylethylamine	<0.1	-	0.4	0.1
Putrescine	39.5 a	2.0	14.5 b	0.7
Cadaverine	0.7 a	0.1	0.4 b	0.1
Histamine	9.2 a	0.5	2.1 b	0.1
Tyramine	5.0 a	0.3	0.4 b	0.1
Spermidine	1.3 a	0.1	0.5 b	0.1
Spermine	0.2	0.1	0.1	0.1

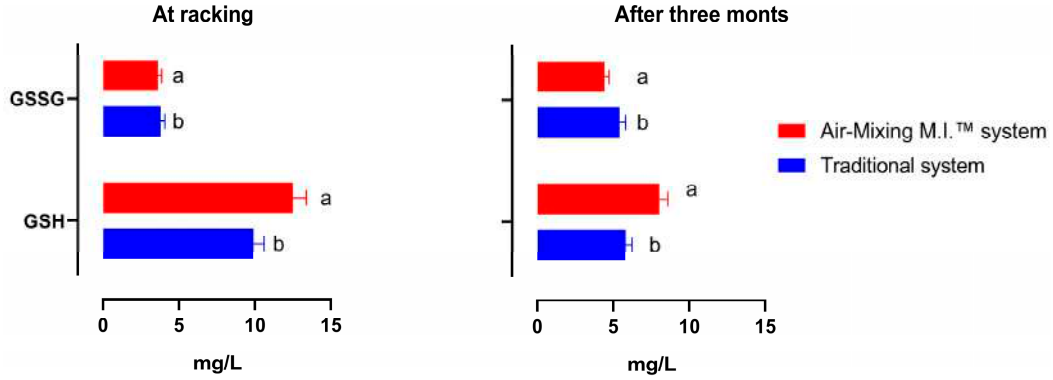

Figure 6. Concentration of reduced (GSH) and oxidized glutathione (GSSG) at racking and after three months of ageing in stainless-steel vessels. Different letters indicate statistically significant differences (*t*-Test, $p < 0.05$).

Analyses to determine the anthocyanins and non-anthocyanin phenolic profiles of the two experimental wines at the racking were carried out. Twelve anthocyanin derivatives were identified in both wines (Table 5). The wine obtained with the traditional system showed a significantly higher content of delphinidin-3-*O*-glucoside, petunidin3-*O*-glucoside, peonidin-3-*O*-glucoside, and malvidin-3-*O*-glucoside, whereas cyanidin-3-*O*-glucoside was detected at low levels in both wines (Table 5). The concentrations of acylated anthocyanins (acetylated and coumaroylated) were higher in the wine produced with the traditional system, while vitisin concentration was similar in the two wines. The concentration of non-anthocyanin phenolic compounds, such as hydroxybenzoic acids, stilbenes, or flavan-3-ols, was the same in the two wines (Table 6). On the contrary, total hydroxycinnamic acid concentration was significantly higher in the wine obtained with the traditional system, mainly because of the higher concentration of the compounds with a dihydroxybenzoic cycle, such as caffeic and caftaric acid. This aspect may be relevant in wines produced without sulphites added. Indeed, caffeic and caftaric acids are the hydroxycinnamic acids most sensitive to oxidation [24]. Both flavonol and phenolic alcohol contents were significantly lower in the wine obtained with the Air-Mixing M.I.™ system, and the most significant differences concerned the concentrations of tyrosol, myricetin, and quercetin.

Table 5. Anthocyanin content (mg/L) in wines obtained with the traditional system and with the Air-Mixing M.I.™ system at racking (s: statistically significant difference; ns: not statistically significant difference, *t*-Test, $p < 0.05$).

mg/L	Air-Mixing M.I.™ System	Traditional System	*t*-Test ($p < 0.05$)
Delphinidin-3-*O*-glucoside	8.0	16.8	s
Cyanidin-3-*O*-glucoside	6.3	7.5	ns
Petunidin-3-*O*-glucoside	14.0	26.3	s
Peonidin-3-*O*-glucoside	9.8	14.0	s
Malvidin-3-*O*-glucoside	95.8	131.6	s
Total Anthocyanin-3-*O*-glucosides	133.9	196.1	s
Peonidin-3-*O*-acetylglucoside	2.7	7.6	s
Malvidin-3-*O*-acetylglucoside	9.5	15.2	s
Total acetylated anthocyanins	12.2	22.8	s
Peonidin-3-*O*-coumaroylglucoside	0.3	0.5	ns
Malvidin-3-*O*-coumaroylglucoside	1.2	3.3	s
Total coumaroylated anthocyanins	1.5	3.8	s
Vitisin A	0.8	0.7	s
Vitisin B	0.2	0.2	ns

Table 6. Non-anthocyanin phenolic compounds (mg/L) in wines obtained with the traditional system and with the Air-Mixing M.I.™ system at racking (s: statistically significant difference; ns: not statistically significant difference, *t*-Test, $p < 0.05$).

mg/L	Air-Mixing M.I.™ System	Traditional System	*t*-Test ($p < 0.05$)
Hydroxybenzoic acids and der.			
gallic acid	43.4	45.9	ns
protocatechuic acid	10.1	9.3	ns
vanillic acid	14.0	14.8	ns
syringic acid	4.0	1.4	s
methylgallate	3.6	4.3	ns
ethylgallate	10.9	14.2	s
Total hydroxybenzoic acids	86.0	89.8	ns
Hydroxycinnamic acids and der.			
trans-caffeic acid	2.3	3.0	s
trans-p-coumaric acid	2.7	3.1	s
cis-p-coumaric acid	0.5	0.5	ns
ferulic acid	0.0	0.0	ns
fertaric acid	0.0	0.0	ns
trans-caftaric acid	12.5	31.3	s
cis-p-coutaric acid	3.0	4.0	s
trans-p-coutaric acid	2.5	8.3	s
Total hydroxycinnamic acids	23.5	50.2	s
Stilbenes			
trans-piceid	9.6	9.7	ns
cis-piceid	0.1	0.4	s
trans-resveratrol	0.4	0.3	s
cis-resveratrol	0.0	0.0	ns
Total stilbenes	10.1	10.4	ns
Flavan-3-ols			
catechin	26.8	28.8	ns
epicatechin	29.6	32.8	ns
epicatechin-3-gallate	34.2	35.7	ns
Total flavan-3-ols	90.6	97.3	ns

Table 6. *Cont.*

mg/L	Air-Mixing M.I.™ System	Traditional System	*t*-Test ($p < 0.05$)
Flavonols			
myricetin-3-glucuronide	2.5	3.9	s
myricetin-3-galactoside	0.7	0.8	s
myricetin-3-glucoside	0.4	0.3	s
quercetin-3-galactoside	0.7	0.0	s
quercetin-3-glucuronide	4.1	4.7	ns
quercetin-3-glucoside	0.1	0.0	s
kaempferol-3-glucoside	0.0	0.0	ns
myricetin	1.3	4.5	s
quercetin	3.1	6.0	s
kaempferol	0.4	0.6	s
Total flavonols	13.3	20.7	s
Phenolic alcohols			
tyrosol	18.2	43.7	s
tryptophol	26.4	26.4	ns
Hydroxytyrosol	4.9	6.0	s
Total phenolic alcohols	49.5	76.1	s

Table 7 shows the total concentrations of free and polymeric anthocyanins and phenolic compounds found in the two wines at the raking and after three months of ageing. The contents of polymeric anthocyanin and polymeric phenolic compounds were higher in the wine obtained with the Air-Mixing M.I.™ system, while the free anthocyanin and phenolic compounds were lower than in the wine obtained with the traditional system. In any case, during the ageing of both wines, the content of free phenolic compounds diminished significantly, whereas that of polymeric compounds increased. Figure 7 shows the differences between the various classes of phenolic compounds in both wines highlighting the lower values the wine obtained with the Air-Mixing M.I.™ system. This result indicates higher phenolic stability in the wine obtained with the Air-Mixing M.I.™ system than in wine obtained with the traditional system.

To evaluate the microbiological stability of the two experimental wines after three months of ageing, yeasts as well as lactic and acetic bacteria were quantified (Table 8). Significant differences were found for the *O.oeni* cell concentrations. Despite the persistence of this indigenous lactic acid bacteria population, no significant increase in biogenic amine concentration was observed.

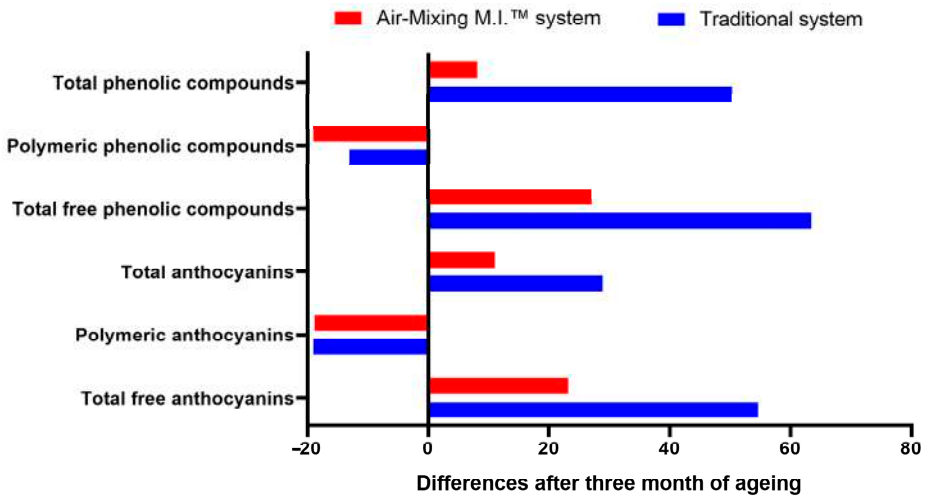

Figure 7. Differences in the concentrations of the various classes of phenolic compounds after the three-month ageing of the two experimental wines.

Table 7. Total free and polymeric anthocyanins and phenolic compounds (mg/L) in wines obtained with the traditional system and with the Air-Mixing M.I.™ system at racking and after three months of ageing (s: statistically significant difference; ns: not statistically significant difference, *t*-Test, $p < 0.05$).

	Air-Mixing M.I.™ System	Traditional System		Air-Mixing M.I.™ System	Traditional System	
mg/L	**At Racking**		***t*-Test ($p < 0.05$)**	**After 3 Months**		***t*-Test ($p < 0.05$)**
Total free anthocyanins	148.6	223.6	s	125.4	169.0	s
Polymeric anthocyanins	17.9	11.0	s	36.7	30.0	s
Total anthocyanins	166.5	234.6	s	155.4	205.7	s
Total free phenolic compounds	273.0	344.6	s	245.9	281.2	s
Polymeric phenolic compounds	44.4	28.3	s	63.4	41.4	s
Total phenolic compounds	317.4	372.9	s	309.2	322.6	ns

Table 8. Microbiological parameters of the wines obtained with Air-Mixing M.I.™ or traditional system after the raking. Different letters in the same row indicate statistically significant differences (*t*-Test, $p < 0.05$).

	Air-Mixing M.I.™ System		Traditional System	
	Mean	**SD**	**Mean**	**SD**
S. cerevisiae (CFU/mL)	45	7	95	7
O. oeni (CFU/mL)	2.73×10^6 a	0.50×10^6	6.63×10^5 b	0.32×10^5
Acetic Acid Bacteria	50	2	60	1
Brettanomyces spp. (CFU/mL)	<10	-	<10	-

3.4. Sensorial Analysis of the Experimental Wines

After three months of ageing, the two experimental wines were subjected to sensorial analysis according to the OIV method (Resolution OIV/CONCOURS 332A–2009) [22]. The mean scores obtained for each descriptor are reported in Figure 8. Statistically significant differences were found only for the gustatory quality descriptor and for the total score obtained as the sum of the scores obtained for all the descriptors considered.

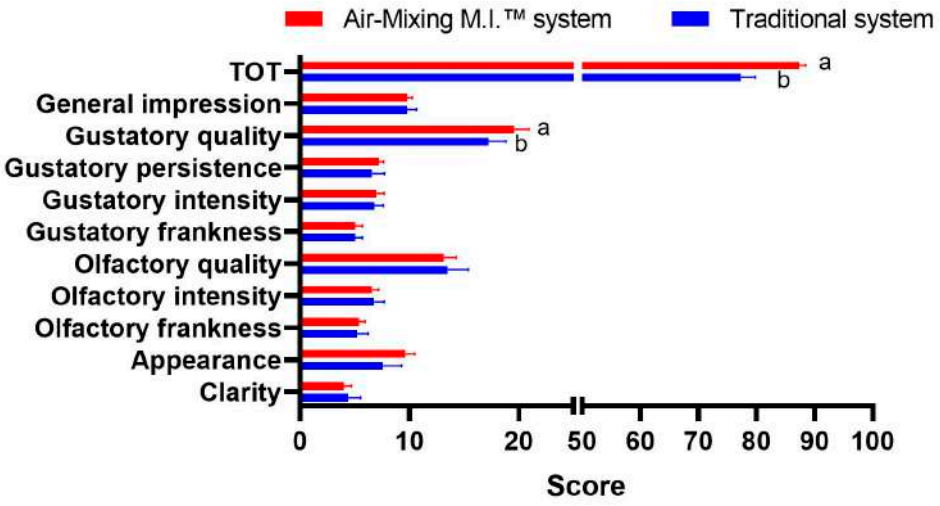

Figure 8. Scores obtained by subjecting the two experimental wines to sensory analysis according to the OIV method (Resolution OIV/CONCOURS 332A–2009. Different letters indicate statistically significant differences (*t*-Test, $p < 0.05$).

4. Discussion

To produce wines without added sulphites, it is necessary to have a technological approach capable of keeping under control the natural production of SO_2 by the yeasts, the oxidation of the phenolic component by the oxygen present in winemaking, and finally, the

microbiological risks linked to the absence of an effective antimicrobial agent. In this study, we evaluated the possibility of producing wines without sulphites added by using low-SO_2-producing *S. cerevisiae* strains to conduct the alcoholic fermentation, microbiological controls in the critical points of the process, and the Air-Mixing M.I.™ system, a technology capable of giving the wine a high extraction of antioxidant substances and an adequate macro-oxygenation simultaneously [12]. Therefore, SO_2-free vinifications, using a low-SO_2-producing *S. cerevisiae* strain, were carried out both with the Air-Mixing M.I.™ system and the traditional method to compare physicochemical, microbiological, and sensory parameters. As regards alcoholic fermentation kinetics, no significant differences were found between the two systems. The fermentation courses in both systems were optimal and practically identical in terms of the yeast oenological performance. This result is not in agreement with the study by Pettinelli et al. [12], in which wine fermentation with air mixing was the fastest one. This different result is probably due to the specific strain capabilities of the used *S. cerevisiae* strain. As expected, both wines at the end of alcoholic fermentation did not show the presence of SO_2, confirming the appropriate choice of the yeast strain used as a starter culture.

At the racking, the free amino acid concentration was higher in the wine obtained with Air-Mixing M.I.™ than in the traditional wine, ascribable to greater yeast autolysis observed in the first wine compared to the second one. During malolactic fermentation, this phenomenon led to a higher formation of the biogenic amines in the wine obtained with Air-Mixing M.I.™. As reported in the literature, biogenic amine formation is attributable to the contemporary presence of lactic acid bacteria with amino acid decarboxylase activity and of the precursor amino acids [25,26]. Therefore, the use of commercial starters to conduct malolactic fermentation in the wine obtained with Air-Mixing M.I.™ is recommended to reduce the risk of the formation of biogenic amines by indigenous lactic acid bacteria.

The most significant differences between the two wines were found in the concentrations of anthocyanin and phenol compounds. In particular, the content of free phenol compounds and anthocyanins was higher in the wine obtained with the traditional system. Considering the influence of temperature on the extraction of these compounds from grape skins [27], this phenomenon could be attributed to the very high temperature at which the cap is usually found during alcoholic fermentation in the traditional method. Indeed, although the temperature of both vessels was set between 26 and 30 °C, only in the Air-Mixing M.I.™ system was the temperature uniform throughout the inside of the tank, as confirmed by the consistent measures at the top and the bottom of the vessel (Figure 3). This uniformity was due to the continuous movement of the mass, which prevented the formation of the cap when the over-pressure valve was opened in the Air-Mixing M.I.™ system. On the contrary, in the traditional system, the temperature was maintained at 26–30 °C only in the lower section of the vessel, whereas in the upper part, especially under the grape skin cap, the temperature rose significantly above 30 °C. These high temperatures, reached during traditional vinification, could favour the extraction of free anthocyanins and free phenolic compounds, although other authors underlined the importance of a uniform temperature to obtain a greater extraction of polyphenols [28].

Unlike the free phenolic and anthocyanin compounds, the polymerized ones were higher in the wine obtained with the Air-Mixing M.I.™ system, suggesting that this technology might favour the wine stabilization. The optimal conditions favouring polymerization are an anthocyanin/tannin ratio of 1:4 and the presence of oxygen [29]. The ideal wine stabilization would be that the polymerization of these compounds occurred simultaneously with their solubilization from the skin grape. However, the phase of the highest extraction of anthocyanins does not coincide with that of tannins and generally occurs when the oxygen is consumed by the yeasts or by the enzymatic systems of the grape. The macro-oxygenation, furnished in Air-Mixing M.I.™ vinification from the second day of alcoholic fermentation until the end of fermentable sugars, was probably the cause of the higher polymerization between tannins and anthocyanins observed compared to the traditional wine. In any case, acetaldehyde produced by the yeasts during the exponential

growth phase does not bind to the SO_2, which is absent, thus being able to participate in the polymerization reactions.

Another interesting aspect of the wine obtained with the Air-Mixing M.I.™ system was the higher concentration of reduced glutathione than the traditional wine, as determined both upon racking and after three months of ageing. This compound, which is present in grapes and can be produced by *S. cerevisiae* during alcoholic fermentation at different concentrations, may be useful in the production of sulphite-free wines because of its ability to control oxidative spoilage damage due to its high antioxidant power [23]. Indeed, according to the current OIV resolutions [30], the addition of glutathione to musts and wines up to a maximum level of 20 mg/L is allowed to limit the intensity of oxidation phenomena, and the use of active yeast strains able to increase the GSH content in wine is also recommended [23].

5. Conclusions

The production of SO_2-free wines is an increasingly sought-after goal by wineries. This study demonstrated how this can be achieved with an adequate choice of a non-sulphur-producing strain and with the use of the Air-Mixing M.I.™ system associated with macro-oxygenation for the aspects related to colour stabilization. However, some critical issues have been highlighted, such as the risk of biogenic amine formation following the autolysis of yeasts at the end of alcoholic fermentation. Therefore, combining this method with selected lactic acid bacteria starter, adequate microbiological controls in the critical points of the winery process, and finally, the wine filtration at the end of the malolactic fermentation, are necessary. However, to ensure the proposed approach is reliable, testing it over several years using grapes of various cultivars, tanks larger than 50 hL, and different fermentation temperatures is advisable.

Author Contributions: Conceptualization, S.G. and L.G.; formal analysis, S.M., D.B., E.M., D.G. and G.B.; validation, D.B. and G.B.; investigation, S.M., D.B., E.M. and S.G.; writing—original draft preparation, S.G. and S.M.; writing—review and editing, L.G. and V.G.; visualization, S.G. and L.G.; funding acquisition, S.G. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by DEFR 2020—Progetto Regionale 6 "Sviluppo Rurale e Agricoltura di qualità"—Interventi a sostegno dei processi di innovazione organizzativa e di processo produttivo nel settore della cooperazione agricola e nei consorzi forestali; contribution of Regione Toscana. Project title "Utilizzo di un sistema innovativo per produrre vini senza l'aggiunta di solfiti—NOSO2". This research was also founded by "bando FABER 4 di Fondazione CR Firenze, Confindustria Firenze e Fondazione per la Ricerca e l'Innovazione, promossa dall'Università degli Studi di Firenze e dalla Città Metropolitana di Firenze".

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The authors would like to thank Terre dell'Etruria Società Cooperativa Agricola and Parsec srl for their technical support during the trials.

Conflicts of Interest: The authors declare no conflict of interest.

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