

Article

Physiological, Genetic, and Fermentative Traits of *Oenococcus oeni* Isolates from Spontaneous Malolactic Fermentation in Koshu Wine

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Abstract

Koshu wine, produced from the indigenous Japanese grape *Vitis vinifera* L. cv. Koshu exhibits a lower pH than other white wines, hindering malolactic fermentation (MLF) by lactic acid bacteria (LAB). Here, we aimed to isolate LAB strains capable of performing MLF under these challenging conditions to improve wine quality. Sixty-four *Oenococcus oeni* and one *Lactobacillus hilgardii* strain were isolated from Koshu grapes and wines that had undergone spontaneous MLF. MLF activity was assessed under varying pH, SO₂, and ethanol conditions in modified basal medium (BM) and Koshu model wine media. Expression of stress-related genes was analyzed using real-time PCR. Carbon source utilization was evaluated via API 50CH assays. All isolates degraded malic acid and produced lactic acid at 15 °C and pH 3.2 in BM without reducing sugars. Seven strains, all identified as *O. oeni*, demonstrated MLF activity at pH 3.0 in modified BM lacking added reducing sugars or tomato juice. Six wine-derived strains tolerated up to 12% ethanol, whereas the grape-derived strain was inhibited at 10%. In a synthetic Koshu wine model (13% ethanol, pH 3.0), wine-derived isolates exhibited higher MLF activity than commercial starter strains. In high-performing strains, *mleA* was upregulated, and most isolates preferred fructose, arabinose, and ribose over glucose. These findings suggest that indigenous *O. oeni* strains from Koshu wine possess unique stress tolerance and metabolic traits, making them promising candidates for region-specific MLF starter cultures that could enhance Koshu wine quality and terroir expression.

Keywords: Koshu wine; malolactic fermentation; *Oenococcus oeni*; spontaneous fermentation



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1. Introduction

Koshu grapes are the only large pale red-purple grape variety indigenous to Japan. In 2010, Koshu became the first Japanese grape variety to be registered with the Organisation Internationale de la Vigne et du Vin (OIV), a milestone that not only recognized its uniqueness but also supported the expansion of Koshu wine exports [1]. However, Koshu wines have a mediocre flavor and lack distinctiveness; therefore, the demand for improved wine quality has grown to meet consumer expectations. Consequently, various advanced winemaking techniques, such as low-temperature fermentation, barrel fermentation and storage, sur lie, carbonic maceration, and the use of inert gas, have been introduced [2,3].

Traditionally, Koshu grapes are cultivated using a trellising system [4]. However, in recent decades, hedge-trimming methods, which are commonly used for European vinifera grapes, have been adopted. Misawa et al. [5] introduced this method for Koshu grape vineyards, demonstrating that the hedging method could produce Koshu grapes with elevated sugar content and a favorable malic/tartaric acid ratio, making them more suitable for vinification. Consequently, efforts were made to explore malolactic fermentation (MLF)—a key process in winemaking involving lactic acid bacteria (LAB) [6,7]—as a strategy to enhance the complexity and overall quality of Koshu wine.

During MLF, LAB convert L-malic acid to L-lactic acid in wine, which improves wine quality by reducing acidity and imparting a buttery and mellow aroma. Typically occurring during or after alcoholic fermentation (AF), MLF is influenced by several factors, including SO₂ concentration, pH, ethanol concentration, temperature, dominant yeast, and micronutrient availability [6]. The primary LAB involved in wine MLF is *O. oeni*, which is known for its tolerance to ethanol and low pH. However, in white wines, particularly those with low pH, MLF is generally more difficult to achieve due to harsher growth conditions and a lack of nutrients for *Lactobacilli* to grow [8].

Onda et al. reported that the free-run juice pH of Koshu grapes harvested in 2014 ranged from 3.07 to 3.31, and in 2015 from 3.19 to 3.31 [9]. Earlier work by Nonomura and Obara [10] identified Koshu as the least likely among seven varieties to undergo spontaneous MLF under natural conditions—with no sugar, starter yeast, or SO₂ supplementation. Nevertheless, more recent observations have contradicted this assumption. In 2017, Chuo Budoshu Co., Ltd. (Koshu, Yamanashi, Japan) confirmed the spontaneous MLF for the first time in wines made from Koshu grapes grown in its vineyard and confirmed its recurrence and stability (internal data). However, attempts made in previous studies failed to isolate *O. oeni* strains from soils and fruits in Koshu grape vineyards throughout the Yamanashi Prefecture [11]. These contradictory observations suggest the existence of a previously overlooked LAB strain that is stress-resistant and may be naturally adapted to the unique environment of Koshu wine.

In this study, we aimed to isolate LAB from Koshu grapes and wines where spontaneous MLF had occurred and to identify these strains at the genetic level. Furthermore, we evaluated the tolerance of the isolated strains to key stress factors, including low pH, SO₂, and ethanol, and compared their MLF performance with that of commercial strains. Finally, to elucidate potential mechanisms underlying their stress resilience, we investigated the expression of stress-response genes and assessed their preferred carbon sources.

2. Materials and Methods

2.1. Strain Isolation and Growth Conditions

LAB were isolated from pre-bottled Koshu wines vinified at the winery (Akeno-cho, Hokuto, Yamanashi, Japan) in 2017 and 2018 (Table 1) and from Koshu grapes collected directly from the vineyard (Akeno-cho, Hokuto, Yamanashi, Japan) just before harvest in 2019. The 2017 and 2018 wines were vinified from grapes harvested at the same vineyard in each respective year and processed as described previously [5]. Briefly, grapes were transported to the winery immediately after harvest, and the whole cluster was pressed to obtain grape juice without adding sulfites. Fermentation was carried out using a commercial dry yeast strain. Upon completion of AF, the must was dispensed into barrels, stainless tanks, and wine bottles for maturation [5]. We collected the samples from fizzy barrels under aging for LAB isolation and organic acid analysis.

Table 1. Comparison of organic acid composition and MLF intensity of Koshu wine in 2017 and 2018.

Vintage Year	Sample Name	MLF *	Malic Acid	Lactic Acid	Citric Acid	Acetic Acid	Tartaric Acid	Succinic Acid
			(g/L)	(g/L)	(g/L)	(g/L)	(g/L)	(g/L)
2017	Koshu wine A (2017-A)	+	1.62	0.60	0.16	0.38	2.56	0.78
2017	Koshu wine B (2017-B)	+	0.31	1.58	0.09	0.44	2.47	0.78
2017	Koshu wine C (2017-C)	---	2.12	0.15	0.17	0.26	2.65	0.87
2018	Koshu wine A (2018-A)	++	0.22	1.27	nd	0.31	2.33	0.67
2018	Koshu wine B (2018-B)	++	nd	1.35	nd	0.23	2.45	0.71
2018	Koshu wine C (2018-C)	---	1.72	nd	0.15	0.19	2.07	0.7

nd: not detected. For each vintage, sample A is the barrel-stored wine, sample B is the bottled wine for quillage, and sample C is the SO₂-added wine after alcohol fermentation. *: ++: 0 g/L < malic acid < 0.3 g/L, +: 0.3 g/L ≤ malic acid ≤ 1.0 g/L, ---: 1.0 g/L ≤ malic acid.

For isolation of LAB from grapes, the bunches were collected directly from the vineyard of the same company using sterile gloves and plastic bags in 2019. Three berries from each cluster were aseptically mashed using a sterile mortar and pestle and diluted with saline. Similarly, Koshu wine and grape must were diluted 10⁰ to 10²-fold and plated onto a basal medium (BM) agar plate containing 10 g glucose, 5 g fructose, 5 g yeast extract, 5 g tryptose, 20 g polypepton, 0.2 g Tween 80, 1 g L-malic acid, 0.08 g MnCl₂·4H₂O, 250 mL tomato juice, 750 mL distilled water, and 10 g agar, supplemented with 1.5% calcium carbonate and 0.25% of kabicidin. Plates were incubated anaerobically at 30 °C for 2 weeks. Colonies forming clear zones were isolated and purified. Gram-positive, catalase-negative bacteria were suspended in 20% glycerol (*v/v*, Fujifilm Wako Pure Chemical Corp., Osaka, Japan) solution and stored at −80 °C until further use [10].

2.2. Identification of Bacteria

2.2.1. Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-TOF MS) Analysis

Rapid and tentative identification of bacterial isolates was performed using a MALDI-TOF mass spectrometer (Shimadzu, Kyoto, Japan). The isolates were cultivated for 2 days on a BM agar plate at 37 °C, and one loop of a colony (approximately 10 mg) was suspended in 300 µL of distilled water and 900 µL of ethanol. A 0.5 µL of the resulting suspension was spotted on a target plate (Shimadzu) and left to dry at 25 °C for 3 min. Each dried spot was overlaid with 1 µL of α-cyano-4-hydroxycinnamic acid matrix solution (Shimadzu) and air-dried at 25 °C before analysis. Mass spectra of each isolate were automatically acquired using an AXIMA Performance Mass Spectrometer (Shimadzu). Tentative species-level identification of unknown bacterial isolates was performed using SARAMIS software v4.10 (AnagnosTec, Potsdam, Germany), which compares the sample spectra against reference spectra available in the in-house library database of the Institute of Enology and Viticulture, University of Yamanashi. Each isolate was identified based on the closest spectral match, with the highest confidence score determining the assigned species [12].

2.2.2. 16S rRNA Gene Sequencing

Genomic DNA was extracted with PrepMan[®] Ultra Reagent (Applied Biosystems, Tokyo, Japan) according to Chen et al. [13,14]. For species identification, the 16S rRNA gene was amplified using the universal primer pair 9F (GAGTTTGATCCTGGCTCAG) and 1492R (AAGGAGGTGATCCAGCC). PCR products were purified and sequenced using a

commercial sequencing service (Fasmac Co., Kanagawa, Japan). The resulting sequences were compared to those in the DNA Data Bank of Japan (DDBJ; <http://www.ddbj.nig.ac.jp>) using NCBI BLAST version 2.8.1 to determine the closest taxonomic match based on sequence similarity [15].

2.3. Preparation of Biomass

Following pre-cultivation, LAB isolates were centrifuged at $12,000 \times g$ for 10 min at 4 °C. The pellets were washed twice with sterile 0.7% saline water and resuspended in sterile water. The resulting suspension was adjusted to an initial concentration of 1.0×10^6 cells/mL for use as the inoculum.

2.4. Fermentation in BM w/o Reducing Sugars

MLF was carried out in 5 mL of BM or modified BM (composition: 20 g polypepton, 0.2 g Tween 80, 1 g L-malic acid, 0.08 g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 250 mL tomato juice, and 750 mL distilled water). The pH was adjusted to either 3.0, 3.2, or 3.4 using 0.1 N NaOH, and samples were incubated at 15 °C. After 7 days, L-malic acid and L-lactic acid concentrations were quantified using HPLC, as described in Section 2.6. The control strains used were MBR PN4 and MBR 31 (Lallemand Inc., Montreal, QC, Canada), which are representative commercial starter cultures previously used in wineries. All experiments were performed in duplicate.

2.5. Fermentation in Modified BM Without Reducing Sugars and Tomato Juice

To evaluate pH tolerance of selected isolates ($n = 8$) and commercial starter culture LACTOENOS 350 PreAC (PreAC; Laffort, Floirac, France), *O. oeni* strains were inoculated at 2×10^5 cells/mL into a modified BM without reducing sugars and tomato juice (20 g polypepton, 0.2 g Tween 80, 1 g L-malic acid, 0.08 g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, and 1000 mL distilled water). The pH was adjusted to 3.0, 3.2, and 3.4, and fermentation was carried out in 5 mL aliquots incubated at 15 °C for 7 days. All tests were performed in duplicate, and L-malic acid consumption was determined using HPLC, as described in Section 2.6.

Tolerance to pH, ethanol, and SO_2 was further evaluated using the same modified BM under the following conditions: pH 3.0, 3.2, and 3.4; ethanol concentrations of 10, 11, and 12% (v/v); and SO_2 concentrations of 5, 10, and 15 mg/L. Bacterial growth was monitored spectrophotometrically at 660 nm using a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan). L-malic and L-lactic acid concentrations were also determined using HPLC. All assays were performed in duplicate at 15 °C.

2.6. Organic Acid Determination and Bacterial Population Count

Organic acids in the samples were quantified using a Shimadzu LabSolutions HPLC system consisting of a Shim-pack SCR-102H column and an autosampler (AS-2000; Shimadzu, Kyoto, Japan). Samples were diluted (1:10) with ultra-pure water and filtered through a 0.22 μm membrane filter (Shimadzu GLC, Tokyo, Japan). The filtrate was transferred to 1.5 mL vials, and 20 μL of each sample was injected into the column at a flow rate of 0.8 mL/min. Quantification was performed using calibration curves generated from organic acid standards, with coefficients of determination (R^2) greater than 0.99 [12].

Bacterial population counts (cells/mL) were determined indirectly by measuring the absorbance at 660 nm using a spectrophotometer UV-1800 (Shimadzu, Kyoto, Japan) [16].

2.7. Fermentation Test in Synthetic Kosu Wine Medium

The synthetic wine medium was prepared based on protocols described in previous studies [5,12,17]. MLF was carried out in a synthetic Kosu wine medium comprising 13.0% ethanol, 4 g tartaric acid, 3 g L-malic acid, 0.3 g citric acid, 0.1 g acetic acid, 0.2 g lactic acid,

2 g D-glucose, 2 g D-fructose, 0.2 g NaCl, 1 g $(\text{NH}_4)_2\text{SO}_4$, 2 g K_2HPO_4 , 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 g MnSO_4 , 2 g yeast extract, and 0.6 g proline. The pH was adjusted to 3.0 using 1 N NaOH. Fermentations were performed in triplicate at a volume of 200 mL in 300 mL Erlenmeyer flasks, incubated at 20 °C. Washed bacterial cells were inoculated into each flask at 1×10^8 cells/mL. All tests were performed in triplicate. L-malic concentrations and bacterial population counts in each sample were determined as described in Section 2.6.

2.8. Analysis of *mleA* and *cfa* Expression

Gene expression analysis was performed following the method described by Guardado et al. [18] with minor modifications. Briefly, bacterial cells were harvested by centrifugation at $8000 \times g$ for 3 min at 4 °C and washed twice with 1 mL of Tris-EDTA (TE) buffer. Total RNA was extracted and purified using Nucleospin RNA columns (Macherey-Nagel GmbH & Co., KG, Dueren, Germany), following the manufacturer's instructions. cDNA was synthesized from RNA (10 ng/ μL) using ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan). Real-time PCR was performed using the THUNDERBIRD Next SYBR qPCR Mix (TOYOBO) using a MyGo Mini Real-Time PCR System (IT-IS International Ltd., Middlesbrough, UK). Amplification was carried out with an initial step at 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s, 55 °C for 15 s, and 72 °C for 30 s. The qPCR efficiency was determined using the standard curves for each primer pair. Gene expression levels were analyzed using the comparative critical threshold ($\Delta\Delta\text{CT}$) method, with normalization to the reference genes (Ldh and 16S rDNA). Each result represents the mean of two independent biological replicates, and each sample was analyzed in technical triplicate.

2.9. Characterization of Strains API50

Carbohydrate fermentation profiles were determined using the API 50CHL system (bioMérieux, Marcy l'Etoile, France) following the manufacturer's instructions. Results were recorded after 24 and 48 h of incubation at 30 °C.

2.10. Statistical Analyses

Fermentation tests in synthetic Koschu wine medium and gene expression analyses were performed in triplicate, and results are presented as averages. The means were compared using one-way ANOVA. Statistical analyses were performed using Bell Curve for Excel (Social Survey Research Information Co., Ltd., Tokyo, Japan), and differences with $p < 0.05$ were considered statistically significant.

3. Results

3.1. Changes in the Organic Acid Composition of Koschu Wines by MLF

Table 1 summarizes the organic acid profiles and the extent of MLF, expressed as intensity, in 2017 and 2018 Koschu wines used for LAB isolation. The wine samples were categorized as A (barrel-stored), B (bottled makeup wine), and C (stainless-steel tanks).

In 2017, malic acid levels were 1.62 g/L in 2017-A, 0.31 g/L in 2017-B, and 2.12 g/L in 2017-C. The moderately low malic acid and slightly elevated lactic acid content in 2017-A, along with observed CO_2 bubbles during barrel storage, suggest that partial MLF had occurred or was underway. The low malic acid concentration in 2017-B indicates that MLF was nearly complete. Similarly, in 2018, malic acid concentrations in samples 2018-A and 2018-B were both around 0.22 g/L, indicating a stronger and nearly complete MLF in these wines. In contrast, malic acid levels in 2017-C and 2018-C; 50 mg/L of total SO_2 was added post-AF, ranged from 1.72 to 2.12 g/L, with lactic acid below 0.15 g/L, indicating that MLF did not occur in these stainless-steel tank samples.

3.2. Isolation and Identification of LAB Isolated from Koshu Wines and Grapes

LAB were isolated from 2017 and 2018 Koshu wines and 2019 grape samples using BM agar plates. A total of 20, 22, and 18 strains were recovered from the 2017-A, 2018-A, and 2018-B wine samples, respectively (Table 2). Additionally, five strains were isolated from the 2019 grape (2019-GR) samples, while no LAB were detected in the 2017-B, 2017-C, or 2018-C samples. Identification via 16S rRNA gene sequencing and MALDI-TOF MS revealed that all isolates from the wine samples were *O. oeni* strains. Among the 2019-GR isolates, four were identified as *O. oeni* and one as *L. hilgardii*. The *O. oeni* isolate shared 99.5–100% sequence homology with *O. oeni* NBRC 100497^T (AB681195), while the *L. hilgardii* isolate showed 99.8% homology with *L. hilgardii* NBRC 15886^T (AB680989). Although not shown here, multiplex RAPD-PCR (random amplified polymorphic DNA polymerase chain reaction) analysis confirmed that these strains are distinct from the commercial LAB starter cultures previously used at the winery.

Table 2. Number and identification of LAB strains isolated from Koshu wines and Koshu grapes.

Vintage Year	Sample Name *	No. of Isolates	Identification
2017	2017-A	20	<i>O. oeni</i> 20 strains
2017	2017-B	0	-
2017	2017-C	0	-
2018	2018-A	22	<i>O. oeni</i> 22 strains
2018	2018-B	18	<i>O. oeni</i> 18 strains
2018	2018-C	0	-
2019	2019-GR	5	<i>O. oeni</i> 4 strains <i>L. hilgardii</i> 1 strain
(Koshu grape)			
Total		65	<i>O. oeni</i> 64 strains, <i>L. hilgardii</i> 1 strain

*; A: the barrel-stored wine, B: the bottled wine for quillage, C: the SO₂-added wine after alcohol fermentation, GR: Grape.

The pH, titratable acidity, and alcohol content of the wines used for bacterial isolation ranged from 3.36 to 3.37, 7.27 to 7.5 g/L (tartaric acid equivalent), and 11.0 to 11.3%, respectively. These levels of pH and alcohol are not considered to significantly inhibit *O. oeni* growth [6,19].

3.3. Comparison of Malic Acid Degradation and Lactic Acid Production on BM

To assess the MLF activity of each isolate, we examined the relationship between malic acid degradation and lactic acid production in the BM (Figure 1). Glucose and fructose were omitted from the BM, as *O. oeni* can produce lactic acid from sugars.

Malic acid degradation accompanied by lactic acid production was observed in all isolates tested at 15 °C and pH 3.2. However, some isolates produced more lactic acid than predicted by malic acid consumption alone, suggesting that residual sugars in the tomato juice may also have been metabolized. Therefore, subsequent experiments were conducted using a modified BM without reducing sugars or tomato juice. For further analysis, a total of eight strains were selected as representative strains: 17CHKO-ML3 isolated from 2017-A, 18CHKO-MLa20, 18CHKO-MLb3, 18CHKO-MLb5, 18CHKO-MLb6, 18CHKO-MLb11, and 18CHKO-MLb14 isolated from 2018-B, and 19CHKO-GR4 isolated from 2019-GR.

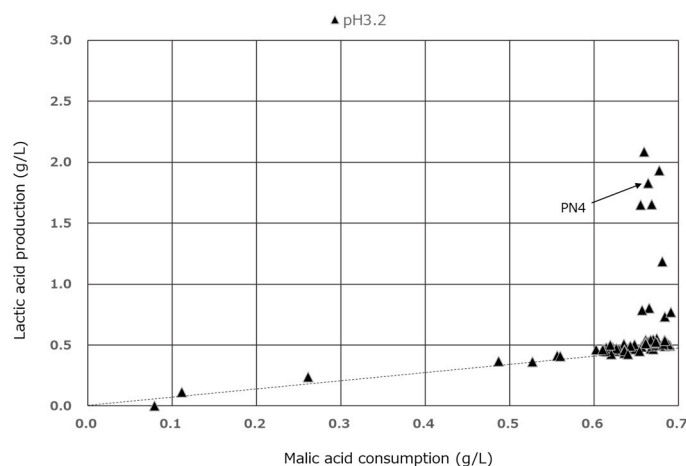


Figure 1. Relationship between malic acid consumption and lactic acid production by the isolated *O. oeni* strains in this study in BM without reducing sugar. The dotted line represents the expected 1:1 molar ratio between malic acid consumption and lactic acid production.

3.4. Effect of pH on MLF Activity in Modified BM

To evaluate the effect of pH on MLF activity, four commercial starter strains known for relatively low pH tolerance—MBR PN4, MBR31, VINIFLORA CH11 (Chr. Hansen, Hørsholm, Denmark), and PreAC—were used as controls alongside the isolates (Figure 2). Although not shown, the molar ratio of malic acid consumption to lactic acid production was approximately 1:1 in all tests conducted in the modified BM. All strains, including PreAC, demonstrated L-malic acid degradation across a pH range of 3.0 to 3.4. Although MLF activity varied among the isolates, seven out of eight strains showed equal or higher activity compared to the PreAC strain, with the exception of 17CHKO-ML3. Furthermore, MLF activity decreased as pH declined in all isolates, and the sole grape-derived isolate, 19CHKO-GR4, maintained relatively higher MLF activity at lower pH values compared to the wine-derived isolates. This likely reflects adaptation to their native environments; acidity increases and pH decreases in grapes, including Koshu, as they mature [19]. Although pH is expected to fluctuate with changes in acidity, immature grapes at an early stage exhibit high acidity and low pH. Therefore, LAB with enhanced tolerance to acid stress should be selected.

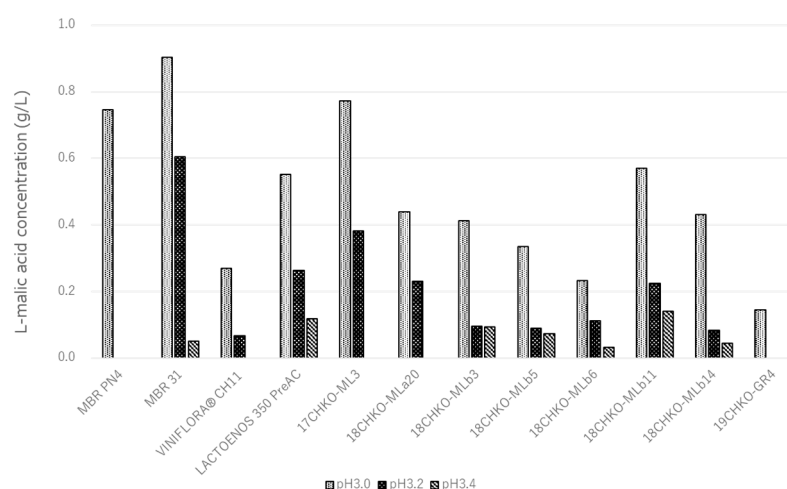


Figure 2. L-malic acid concentration on day 7 post-inoculation in modified BM at different pH levels. 17CHKO-ML3, 18CHKO-MLa20, 18CHKO-MLb3, 18CHKO-MLb5, 18CHKO-MLb6, 18CHKO-MLb11, and 18CHKO-MLb14 strains isolated from wine and 19CHKO-GR4 strain isolated from grapes were used. Commercial starters MBR PN4, MBR 31, VINIFLORA CH11, and PreAC were used as controls. Initial L-malic acid concentration in the culture medium was 1 g/L.

3.5. Effect of Total SO₂ on MLF Activity in Modified BM

The effect of total SO₂ on MLF activity was assessed across the isolates, similarly to the pH experiments (Figure 3). At 10 mg/L SO₂, isolate 19CHKO-GR4 and the commercial starter strain MBR 31 exhibited almost no MLF activity, whereas the other isolates retained some MLF activity. Furthermore, the Koshu wine isolates demonstrated higher MLF activity than all commercial starter strains tested, and total SO₂ concentrations above 25 mg/L significantly inhibited MLF activity in all isolates.

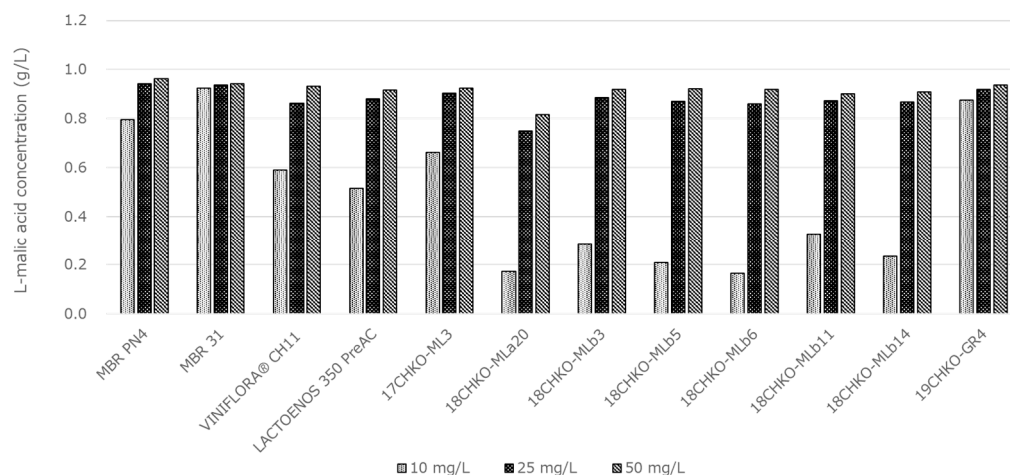


Figure 3. L-malic acid concentration on day 7 after inoculation into the modified BM (pH 3.2) with varying SO₂ concentrations (10, 25, and 50 mg/L). Initial L-malic acid concentration and strains used are consistent with those in Figure 2.

3.6. Effect of Ethanol on MLF Activity in Modified BM

The effect of ethanol on MLF activity was evaluated by adding ethanol at concentrations of 10, 11, and 12% (v/v) to the modified BM (Figure 4). The wine-derived isolates maintained relatively stable MLF activity across this ethanol range. In contrast, PreAC, the commercial starter strain, showed a sharp decline in MLF activity with increasing ethanol, with nearly complete inhibition at 12% ethanol. The grape-derived strain 19CHKO-GR4 exhibited complete inhibition of MLF activity even at the lowest ethanol concentration tested (10%).

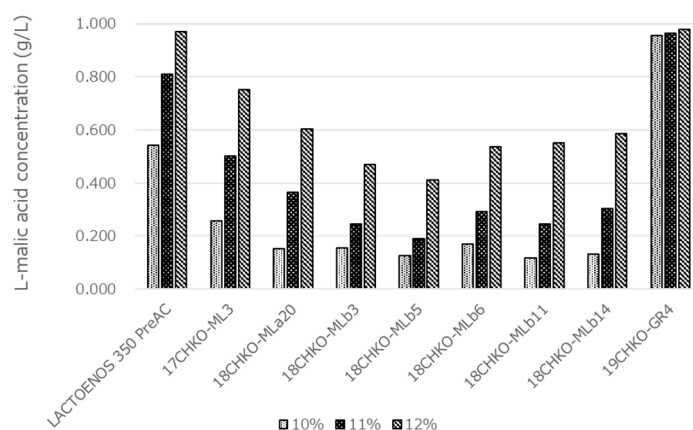


Figure 4. L-malic acid concentration on day 7 after inoculation into modified BM (pH 3.2) under different ethanol concentrations (10, 11, and 12% v/v). The commercial starter PreAC served as a control. Initial L-malic acid concentration was consistent with Figure 2.

3.7. Comparison of MLF Activity in Koshu Model Wine Medium

As some Koshu wines exhibit pH values below 3.0 and alcohol content of 13% or higher, we evaluated whether the isolated strains could perform MLF under these harsh conditions. Koshu model wines were prepared with pH 3.0, 13% ethanol, and an L-malic acid concentration of 3.0 g/L to assess MLF activity (Figure 5). Tested strains included 18CHKO-MLa20, 18CHKO-MLb5, and 18CHKO-MLb14—selected for their high MLF activity under inhibitory conditions in modified BM—along with 19CHKO-GR4 and several commercial starter culture strains. The inoculum was standardized to 1×10^8 cells/mL based on optical density, reflecting bacterial concentration in the model wine medium. Under these conditions, wine-derived isolates consistently demonstrated higher MLF activity than the commercial starter strains, although some variation was observed among individual isolates.

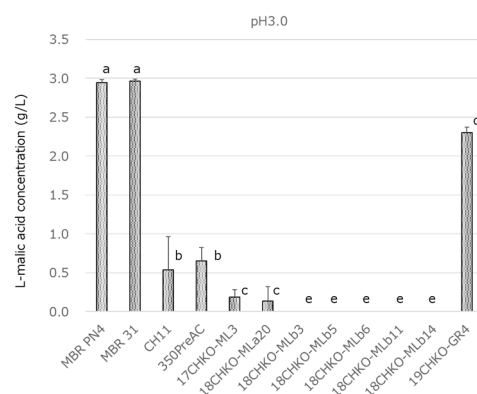


Figure 5. L-malic acid degradation by strains in synthetic Koshu model wine medium (pH 3.0, Alc. 13%). Different letters indicate statistically significant differences between variables. Values with the same letter are not statistically significant ($p < 0.05$).

3.8. Effect of SO₂ Concentration on MLF Activity in Koshu Model Wine Medium

Using the PreAC strain, which exhibited the highest MLF activity among the commercial starter strains, as a control, we evaluated the effect of SO₂ addition on MLF activity in the Koshu model wine medium. While MLF activity was partially maintained at 10 mg/L SO₂ in modified BM, all tested strains showed significant inhibition in the Koshu model wine medium at this concentration. At a SO₂ concentration of 3 mg/L, all strains showed almost the same dynamics as at 5 mg/L, so Figure 6 showed the results for 0 mg/L and 5 mg/L. PreAC did not complete MLF by the seventh day, but the isolated strain completed MLF by the second day, even with the addition of SO₂, as it did without the addition. Although the inhibitory SO₂ concentrations varied between media, the relative comparison among isolates remained consistent.

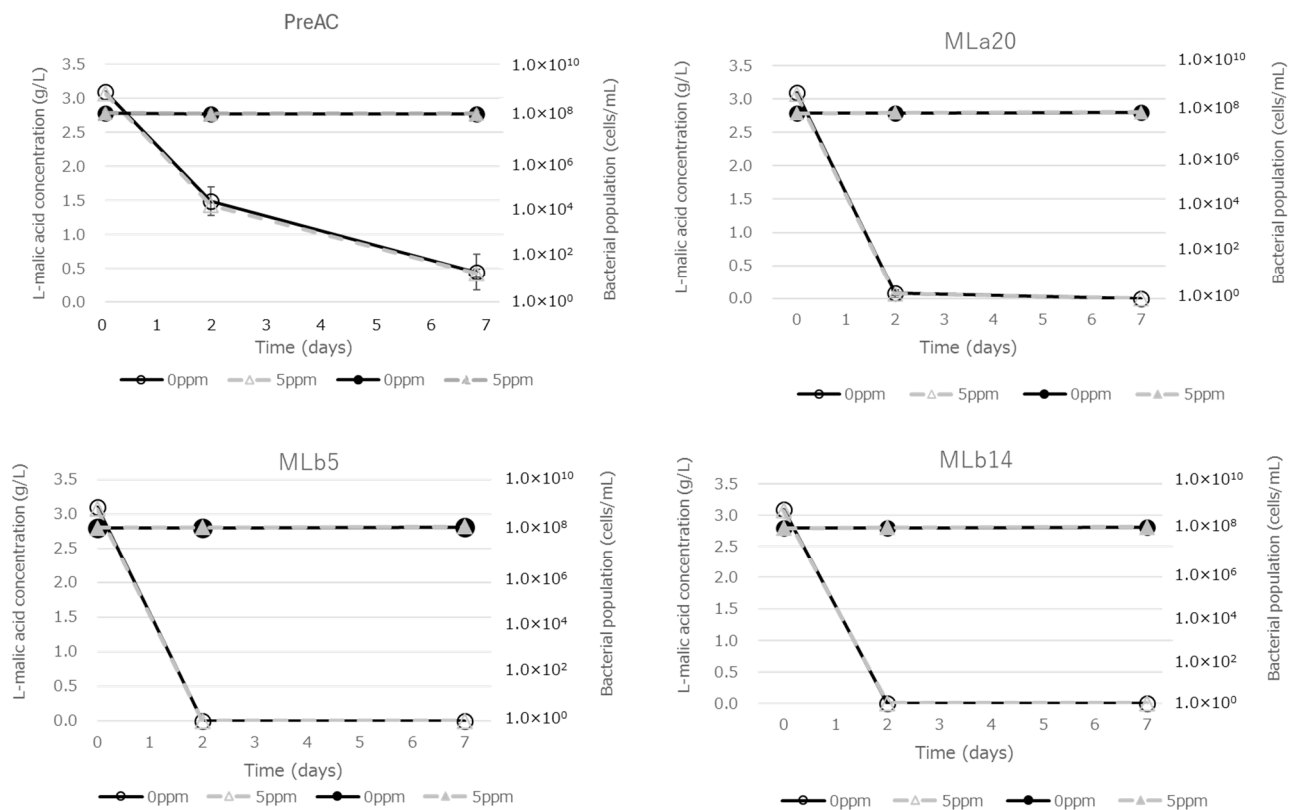


Figure 6. SO₂ tolerance of strains at the synthetic Koshu model wine medium (pH 3.0, Alc. 13%). ○, △: L-malic acid concentration, ●, ▲: bacterial population $p < 0.05$.

3.9. Stress-Related Gene Expression Analysis

In addition to ethanol, various chemical and physical factors—such as low pH, SO₂, malic acid, and temperature—can inhibit the growth of *O. oeni* in the winemaking environment [6]. As the wine-derived isolates thrived under such conditions, it is likely they have developed tolerance mechanisms to withstand these stresses. Previous studies on ethanol tolerance using *O. oeni* strains with enhanced MLF activity under high ethanol conditions (selected by directed evolution) have examined the responses of multiple stress-related genes [20]. In this study, we focused on the expression of *mleA* and *cfa*.

Expression levels of *mleA* and *cfa* were measured on day 2 post-inoculation in model wine medium for the 18CHKO-MLa20, 18CHKO-MLb5, and 18CHKO-MLb14 strains (which had demonstrated high MLF activity) and compared to the commercial PreAC strain. The findings are presented as relative expression levels normalized to PreAC without SO₂ (set to 1) (Figure 7). Real-time PCR confirmed the amplification of both genes under all tested conditions.

For the highly active strains 18CHKO-MLb5 and 18CHKO-MLb14, *mleA* expression was significantly elevated compared to the PreAC and 18CHKO-MLa20 strains, correlating well with their MLF performance. This aligns with Betteridge et al. [20], who reported higher *mleA* expression in ethanol-tolerant *O. oeni* strains than that in parental strains 24 h post-inoculation under high ethanol stress. Conversely, no significant differences in *cfa* expression were observed among the tested strains on day 2 after inoculation, suggesting a distinct regulatory pattern from *mleA*. When 5 mg/L SO₂ was added to the model wine, both *mleA* and *cfa* expression tended to increase across all strains. Among them, 18CHKO-MLb5 and 18CHKO-MLb14 strains exhibited a significantly greater upregulation of these genes relative to 18CHKO-MLa20 and PreAC, indicating a possible role in SO₂ stress tolerance.

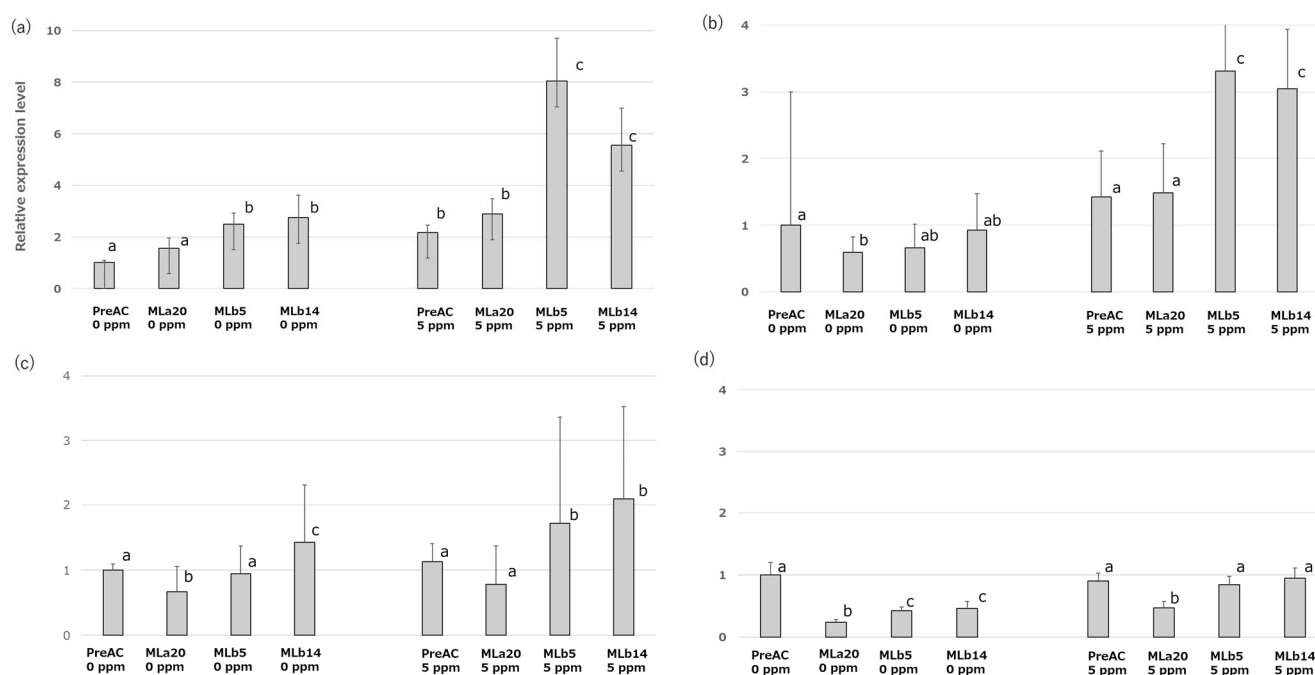


Figure 7. Evaluation of the relative expression of *mleA* and *cfa* on days 2 and 7 of MLF at different sulfite concentrations. (a) day 2, *mleA*; (b) day 2, *cfa*; (c) day 7, *mleA*; (d) day 7, *cfa* expression levels. Values with the same letter are not statistically significant ($p < 0.05$).

3.10. Utilization of Carbon Sources

The carbon source utilization profiles of the isolates were assessed using the API 50CH system (Table 3). All isolates from both wine and grape samples consistently utilized fructose as a carbon source. In addition to fructose, the strains were able to metabolize arabinose, ribose, and esculin but notably did not utilize glucose.

Table 3. Investigation of the carbon source metabolized by the *O. oeni* strains isolated in this study. Strains were used as samples for this test.

Sugars	Strains							
	ML3	MLa20	MLb3	MLb5	MLb6	MLb11	MLb14	GR4
Glycerol	-	-	-	-	-	-	-	-
Erythritol	-	-	-	-	-	-	-	-
D-arabinose	-	-	-	-	-	-	-	-
L-arabinose	-	+	+	+	+	+	+	+
D-ribose	-	-	-	-	-	-	-	+
D-xylose	-	-	-	-	-	-	-	-
L-xylose	-	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-	-
Methyl-β	-	-	-	-	-	-	-	-
D-xylopyranoside	-	-	-	-	-	-	-	-
D-Galactose	-	-	-	-	-	-	-	-
D-Glucose	-	-	-	-	-	-	-	-
D-Fructose	+	+	+	+	+	+	+	+
D-mannose	-	-	-	-	-	-	-	-
L-sorbose	-	-	-	-	-	-	-	-
L-rhamnose	-	-	-	-	-	-	-	-
Dulcitol	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-
D-Mannitol	-	-	-	-	-	-	-	-
D-sorbitol	-	-	-	-	-	-	-	-

Table 3. Cont.

Sugars	Strains							
	ML3	MLa20	MLb3	MLb5	MLb6	MLb11	MLb14	GR4
Methyl- α	-	-	-	-	-	-	-	-
D-mannopyranoside	-	-	-	-	-	-	-	-
Methyl- α	-	-	-	-	-	-	-	-
D-glucopyranoside	-	-	-	-	-	-	-	-
N-acetyl glucosamine	-	-	-	-	-	-	-	-
Amygdalin	-	-	-	-	-	-	-	-
Arbutin	-	-	-	-	-	-	-	-
Esculin	+	+	+	+	+	+	+	+
Salicin	-	-	-	-	-	-	-	-
D-cellobiose	-	-	-	-	-	-	-	-
D-maltose	-	-	-	-	-	-	-	-
D-lactose	-	-	-	-	-	-	-	-
D-melibiose	-	-	-	-	-	-	-	-
D-sucrose	-	-	-	-	-	-	-	-
D-trehalose	-	-	-	-	-	-	-	-
Inulin	-	-	-	-	-	-	-	-
D-melezitose	-	-	-	-	-	-	-	-
D-raffinose	-	-	-	-	-	-	-	-
Starch	-	-	-	-	-	-	-	-
Glycogen	-	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-	-
Gentiobiose	-	-	-	-	-	-	-	-
D-turanose	-	-	-	-	-	-	-	-
D-lyxose	-	-	-	-	-	-	-	-
D-tagatose	-	-	-	-	-	-	-	-
D-fucose	-	-	-	-	-	-	-	-
L-fucose	-	-	-	-	-	-	-	-
D-arabitol	-	-	-	-	-	-	-	-
L-arabitol	-	-	-	-	-	-	-	-
Gluconate	-	-	-	-	-	-	-	-
2-keto-gluconate	-	-	-	-	-	-	-	-
5-keto-gluconate	-	-	-	-	-	-	-	-

Note: + positive reaction; - negative reaction.

4. Discussion

In this study, the samples used to isolate LAB, 2017-A, 2017-B, 2018-A, and 2017-B, suggest that MLF had occurred or was in progress. However, 2017-C and 2018-C did not undergo MLF. Stainless steel tanks were highly airtight, did not allow oxygen to pass through, and allowed temperature control, so MLF did not occur. On the other hand, barrels had high thermal conductivity and allowed moderate oxygen to pass through, providing an environment suitable for the growth of lactic acid bacteria, which is thought to have allowed MLF to progress. As SO₂ was added to samples 2017-C and 2018-C after AF, this suggests that MLF occurred after AF and was inhibited by the addition of SO₂. Furthermore, in samples where MLF occurred (2017-A, 2017-B, 2018-A, and 2018-B), citric acid levels decreased by 0.01 to 0.15 g/L, while acetic acid levels increased by 0.04 to 0.18 g/L compared to wines without MLF. These changes are consistent with LAB metabolizing citric acid [21]. Generally, *O. oeni* growth is inhibited by SO₂ concentrations around 1–10 mg/L [22]. In this study, no SO₂ was added during pressing, and a commercially available *Saccharomyces cerevisiae* strain—known for low to moderate SO₂ production according to the manufacturer—was selected. Although yeast produces SO₂ during AF, *S. cerevisiae* is reported to generate less SO₂ than *Saccharomyces bayanus*. Literature examining the SO₂

production of each of the yeasts currently on the market indicates that some yeast strains produce less than 10 mg/L SO₂, while many produce higher levels [23]. Winemakers often select yeast strains based on the desired wine style, but care should be taken when introducing MLF, as certain yeast and LAB combinations can inhibit MLF [24]. Furthermore, lactobacilli were not isolated from the 2017-B wine despite MLF being observed in that sample. This wine was bottled and stored in a smaller container relative to barrels to avoid potential environmental instability.

The spontaneous MLF was confirmed in Kosshu wines, and 64 strains of *O. oeni* and one strain of *L. hilgardii* were successfully isolated from wine and grape samples. The wine-derived *O. oeni* strains demonstrated superior tolerance to low pH, high ethanol, and SO₂ compared to commercial starter cultures, suggesting that these strains have adapted to the harsh wine environment and possibly acquired specific stress-resistance traits [25]. In this study, four *O. oeni* strains and one *L. hilgardii* strain were isolated from grape samples, suggesting these beneficial LAB may naturally inhabit vineyards and fruit. Franquès et al. [26] reported the isolation of *O. oeni* from Grenache and Carignan grapes but noted *L. plantarum* as the predominant LAB on grapes. To date, *O. oeni* has not been reported from Kosshu grapes or wines, making this the first report of LAB isolated from Kosshu.

We hypothesize that the spontaneous MLF observed in Kosshu wines is primarily driven by LAB originating from vineyards and grape surfaces. The observed differences in ethanol tolerance between wine- and grape-derived isolates support this hypothesis, as grape isolates—lacking prior exposure to ethanol—showed lower tolerance. In contrast, wine-derived strains displayed adaptations consistent with survival and activity in ethanol-rich environments during AF. This aligns with findings by Sumby et al. [27], who reported that *O. oeni* populations increase dramatically from AF to MLF, undergoing genetic variation and environmental selection that yield more robust strains.

Furthermore, all isolated *O. oeni* strains lacked glucose metabolism and utilized a narrow range of carbon sources, such as fructose, arabinose, and ribose. Cibrario et al. [28] analyzed 41 *O. oeni* strains from various wines and ciders of different countries of origin, regions of production, and product types, and examined their growth on 18 wine-related carbohydrates. Most of these strains (>75%) metabolized glucose, trehalose, ribose, cellobiose, mannose, and melibiose, while fructose and L-arabinose were utilized by about half of the strains; nevertheless, all strains utilized glucose. The absence of glucose utilization in the *O. oeni* isolates from Kosshu wine in this study suggests these strains may represent a rare or unique phenotypic variant. It would be valuable to investigate whether this glucose non-utilization is a common trait among *O. oeni* strains from Kosshu vineyards or specific to the isolates from this particular winery. This rare metabolic phenotype diverges from most previously reported *O. oeni* strains and may reflect selective pressures unique to the Kosshu fermentation environment [28].

Gene expression analysis showed that the most MLF-active strains (18CHKO-MLb5 and 18CHKO-MLb14) exhibited significantly elevated *mleA* expression, indicating a molecular basis for their ethanol and SO₂ tolerance. In contrast, *cfa* expression did not differ significantly among strains, suggesting distinct regulatory mechanisms or response timing for this gene. Considering that stress adaptation in *O. oeni* is polygenic, future research should examine additional candidate genes such as *groEL*, *hsp*, and *eno* to further elucidate tolerance mechanisms [29–31]. Furthermore, whole-genome sequencing and comparative genomics could provide deeper insights into the genetic determinants of stress tolerance and the unique phenotypes observed in the Kosshu-derived *O. oeni* strains. While these genomic analyses were beyond the scope of the present study due to time constraints, we recognize their importance and intend to address them in future investigations.

If these indigenous *O. oeni* strains can consistently perform MLF under high-stress wine conditions, they represent promising starter cultures for Koshu and other wines where MLF is challenging. Although accidental MLF has been observed in some Japanese Koshu wines, its commercial application remains limited. Hence, strains capable of inducing rapid, stable MLF are needed. Therefore, the successful isolation of *O. oeni* from Koshu wine for the first time is highly significant, opening opportunities to employ indigenous strains for more efficient MLF than commercial starters. To this end, optimal pre-culture conditions and inoculation strategies must be developed. For example, Onda et al. [32,33] reported successful MLF in Koshu sparkling wine by co-inoculation of selected LAB and yeast strains, completing MLF within 68 days. Although co-inoculation is increasingly gaining traction in winemaking, we propose that sequential inoculation may be more suitable for Koshu wines, especially as all isolates utilize fructose. To minimize competition for sugars with yeast, LAB should be inoculated after AF, or once residual sugar levels are low. Furthermore, using yeast strains with minimal SO₂ production can help reduce stress on LAB [34].

A potential concern is the capacity of these isolates to metabolize citric acid, which can lead to increased production of acetic acid, acetoin, and diacetyl—compounds that affect wine flavor and quality. For instance, diacetyl has a sensory threshold of 0.9 to 2.8 mg/L [19,35]. Moreover, biogenic amine formation must be carefully monitored [36]. Therefore, further studies are warranted to characterize metabolic byproducts and establish control measures, including sensory evaluations and analytical monitoring during fermentation. To enhance practical applicability, data on seasonal variability in Koshu vineyards—such as fluctuations in pH and ethanol—would aid in assessing strain performance under real-world conditions. Additionally, scaling from laboratory- to industrial-scale fermentations introduces challenges such as oxygen gradients, microbial competition, and process variability, which must be addressed for commercial implementation. In summary, future research should focus on optimizing culture conditions, inoculation timing, and compatibility with yeast while ensuring product quality and safety through sensory and biochemical assessments. This study represents a critical step toward leveraging indigenous microbial resources to enhance the terroir expression and overall quality of Koshu wine [34].

5. Conclusions

This study confirmed spontaneous MLF in Koshu wines and successfully isolated 64 strains of *O. oeni* and 1 strain of *L. hilgardii* from wine and grape samples. The wine-derived *O. oeni* strains exhibited superior tolerance to low pH, high ethanol, and SO₂, indicating their adaptation to the wine environment. Gene expression analysis revealed that most MLF-active strains had significantly higher *mleA* expression, suggesting a molecular basis for stress tolerance. Additionally, all isolates lacked glucose metabolism and displayed a unique carbon utilization profile, which is unusual among known *O. oeni* strains. Under low-stress conditions, some isolates completed MLF in as few as 3 days; even under challenging conditions of high alcohol and low pH, most strains completed MLF within 7 days, highlighting their potential as robust starter cultures. These findings indicate that indigenous *O. oeni* strains from Koshu wine are promising candidates for region-specific MLF starter cultures, particularly in challenging fermentation environments. Future research should focus on optimizing inoculation strategies and culture conditions and assessing the sensory and safety parameters of wines produced with these strains. Harnessing native microbial resources has a strong potential to improve both the quality and terroir expression of Koshu wine.

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Abbreviations

The following abbreviations are used in this manuscript:

MLF	Malolactic fermentation
LAB	Lactic acid bacteria
BM	Basal medium
PCR	Polymerase chain reaction
API	Analytical Profile Index
RAPD	Random amplified polymorphic DNA
AF	Alcoholic fermentation

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