

PHENOLIC PROFILE AND ANTIBACTERIAL ACTIVITY OF YOUNG AND AGED WINE FRACTION FROM ARGENTINA

PERFIL FENÓLICO Y ACTIVIDAD ANTIBACTERIANA DE FRACCIÓN DE VINO JOVEN Y AÑEJO DE ARGENTINA

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ABSTRACT

Background: During the winemaking process, the presence of lactic acid bacteria (LABs), principally *Lentilactobacillus* genera, can contribute to the formation of biogenic amines. Phenolic compounds (PCs) are important wine compounds due to their biological activities. **Objectives:** This study qualitatively and quantitatively characterized the low molecular weight fraction of phenolic compounds (LMF) obtained from commercial young (Y) and aged (A) Tannat wines produced in Argentina and evaluated the effect of the LMF on the viability of oenological LABs. **Methods:** The LMF was obtained from dealcoholized wine by liquid-liquid extraction with ethyl acetate. The phenolic profile was determined by LC-DAD-FLD. The effect of the LMF on the viability of three spoilage LABs strains of *Lentilactobacillus hilgardii* (5w, 6F and X1B), that inoculated at concentration of 10^4 or 10^7 cfu/mL, was examined in a synthetic wine-like medium (SWLM) added with LMF-Y and LMF-A. **Results:** The concentrations of the different PCs determined by LC-DAD-FLD showed significant differences, with higher values in LMF-Y (95.1 mg/L) than in LMF-A (61.4 mg/L). LMF-Y and LMF-A inhibited spoilage LAB, reducing viability to "A" values between -0.70 and -0.98 Log cfu/mL. At high inoculum levels, both fractions caused growth arrest, with viability remaining close to initial values. **Conclusions:** The phenolic characterization of LMF-Y and LMF-A was reported for the first time in Argentinean red wine. Additionally, these results provide evidence of the potential applicability of LMF as an antimicrobial agent in winemaking.

Keywords: Wine; Phenolic Compounds, Antibacterial Activity.

RESUMEN

Antecedentes: Durante el proceso de vinificación, la presencia de bacterias ácido lácticas (BAL), principalmente del género *Lentilactobacillus*, puede contribuir a la formación de aminas biógenas. Por otro lado, los compuestos fenólicos (CF) son componentes importantes del vino debido a sus actividades biológicas. **Objetivos:** Este estudio caracterizó cualitativa y cuantitativamente la fracción de compuestos fenólicos de bajo peso molecular (FBP) obtenida de vino comercial Tannat, joven (J) y añejo (A), producidos en Argentina. Además, evaluó el efecto de FBP sobre la viabilidad de BAL enológicas. **Métodos:** La FBP fue obtenida por extracción líquido-líquido con acetato de etilo a partir de vino desalcoholizado. El perfil fenólico se determinó mediante LC-DAD-FLD. Se evaluó el efecto del FBP sobre la viabilidad de tres cepas deteriorantes de *Lentilactobacillus hilgardii* (5w, 6F y X1B), inoculadas a una concentración de 10^4 o 10^7 ufc/mL en un medio sintético similar vino (MSSV) adicionado con FBP-J y FBP-A. **Resultados:** Las concentraciones de los distintos CF mostraron diferencias significativas entre ambas fracciones, siendo mayores en FBP-J (95,1 mg/L) que en FBP-A (61,4 mg/L). FBP-J y FBP-A inhibieron a las bacterias lácticas alterantes, reduciendo la viabilidad a valores de "A" entre $-0,70$ y $-0,98$ Log ufc/mL. A niveles de inóculo alto, ambas fracciones provocaron una detención del crecimiento, manteniéndose la viabilidad cercana a los valores iniciales. **Conclusiones:** Por primera vez, se reportó la caracterización fenólica de FBP-J y FBP-A de vino tinto argentino. Además, estos resultados ponen en evidencia la posible aplicación del FBP como agente antimicrobiano en la vinificación.

Palabras Claves: Vinos, Compuestos Fenólicos, Actividad Antibacteriana.

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INTRODUCTION

LAB species are normally present during the vinification process and can have spoilage or beneficial activity. Some of them can express amino acid-decarboxylase enzymatic activities and may contribute to the formation of undesirable compounds, such as biogenic amines (1). These compounds, particularly histamine, have been reported to affect the health of susceptible consumers (1). In traditional winemaking, sulfur dioxide (SO₂) is used as an additive for its selective antimicrobial effects, particularly against LAB. However, the use of this additive is strictly controlled, as high doses can cause health disorders in consumers and organoleptic alterations in the final product (2,3). Phenolic compounds, natural constituents of grapes and wines, are secondary metabolites. They are primarily synthesized through the pentose phosphate pathway, shikimate and phenyl-propanoid pathways (4), and can be formed and transformed during the winemaking process (5). These compounds include mainly hydrolysable tannins (C-glucosidic ellagitannins), phenolic acids, and phenolic aldehydes.

Currently, some alternatives to the use of SO₂ are being studied mostly based on natural antimicrobial agents. In this regard, studies of the inhibitory activities of diverse classes of phenolic compounds present in wine (benzoic acids, hydroxycinnamic acids, flavan-3ols, stilbenes, and flavonols) against oenological LAB have been carried out (6,7,8,9). In addition, phenolic extracts from different varietal wines have been reported for their antibacterial properties against numerous spoilage LAB (10,6,8,9,11).

The aging process of red wine produces changes in phenolic composition due to contact with oak wood, which involves reactions between diverse types of flavonoids producing copigmentation, cycloaddition, polymerization, and oxidation processes (12,13,14,15,16,17).

In this study, our objectives were to characterize the low-molecular-weight phenolic fraction obtained from commercial young (Y) and aged (A) Tannat wines produced in Tucumán. In addition, we aimed to evaluate the effect of these fractions on the viability of wine-spoilage lactic acid bacteria (LAB) to explore new antibacterial alternatives for the winemaking process.

MATERIAL AND METHODS

Wine samples

Young (Y) and Aged (A) wines of Tannat varietal from the same geographical origins were obtained from Arcas de Tolombón winery (Colalao del Valle, Tucuman, Argentina) during the 2014 vintage. For the aging process, the wine was stored for 6 months in oak barrels at 15-18°C.

Isolation of phenolic compound fraction

The low-molecular-weight fraction of phenolic compounds (LMF) from wine was obtained using a liquid-liquid extraction method previously described by Stivala (7). Ethanol was previously removed from each wine sample by vacuum distillation at 35°C for 20 min, under controlled conditions. Then, an aliquot of 150 mL of the dealcoholized wine was adjusted to pH 2.0 with 10 M HCl using a calibrated digital pH meter, and the mixture was extracted three times with 100 mL of ethyl acetate. The pH was checked after acidification and maintained at pH 2.0 throughout the extraction process. Each extraction procedure was carried out under continuous agitation for 15 min at room temperature. Afterwards, the organic fractions were pooled, dehydrated with 2.5 g of anhydrous Na₂SO₄, and filtered. The solvent was evaporated to dryness under a gentle stream of nitrogen at low temperature to minimize oxidative degradation. The dried extract was subsequently reconstituted to a final volume of 2 mL prior to chromatographic analysis.

Acidic conditions, nitrogen atmosphere, and limited exposure to oxygen were applied throughout the procedure to preserve the stability of phenolic compounds.

Characterization of wine phenolic fraction of low molecular weight

Total phenolic compounds (TPC) of wines and LMFs were measured spectrophotometrically by the Folin-Ciocalteu's (FC) method adapted for micro-techniques by Cicco (18). Briefly, the LMF was first dissolved in 40% (v/v) methanol, and then, 100 µL of correctly diluted samples were pipetted into separate test tubes, which were supplemented with 100 µL Folin-Ciocalteu reagent. After 2 min, 800 µL 5% (w/v) Na₂CO₃ was added. The mixture was stirred and heated to 40°C for 20 min. The absorption of the blue solution was measured at 740 nm. A calibration curve of gallic acid was performed to express the phenolic content as gallic acid equivalent (mEq GAE).

An accurately weighed portion of the low molecular weight fraction of phenolic compounds was analyzed by LC-DAD-FLD (LC), a method previously described by Ferreyra (19). A total of twenty phenolic compounds, including flavonoids (flavanols, flavonols, and dihydroflavonols) and non-flavonoids (hydroxybenzoic and hydroxycinnamic acids, stilbenes, and phenolic alcohols) were quantified. Separation and quantification of PCs were performed using a Dionex Ultimate 3000 (Dionex Softron GmbH, Thermo Fisher Scientific Inc., Germering, Germany) equipped with a vacuum degasser unit, an autosampler, a quaternary pump, a chromatographic oven, a diode-array (Dionex DAD-3000 (RS)), and a dual-channel fluorescence detector (FLD-3400RS Dual-PMT) connected in series. Separation was performed on a reversed-phase Kinetex C18 column (3.0 mm × 100 mm, 2.6 μm; Phenomenex, Torrance, CA, USA) at 35 °C using water with 0.1% formic acid (solvent A) and acetonitrile (solvent B), and the following gradient program: 0–1.7 min, 5% B; 1.7–10 min, 30% B; 10–13.5 min, 95% B; 13.5–15 min, 95% B; 15–16 min, 5% B; 16–19 min, 5% B. The flow rate was set to 0.8 mL min⁻¹, and the total runtime was 19 min. Injection volumes were 5 μL for standards and wine samples and 1–2.5 μL for LMF. Wavelengths of 254, 280, 320, and 370 nm were used depending on the targeted analytes for DAD, while an excitation wavelength of 290 nm and emission responses monitored at 315, 360 and 400 nm were used for FLD. The identification of TPC-LC in samples was based on the comparison with pure standards.

External calibration was performed using pure standards of the quantified phenolic compounds, including gallic acid, 3-hydroxytyrosol, caftaric acid, (–)-epigallocatechin, (+)-procyanidin B1, (+)-catechin, procyanidin B2, (–)-epicatechin, (–)-epigallocatechin gallate, dihydroquercetin 3-rhamnoside (astilbin), caffeic acid, syringic acid, coumaric acid, (–)-epicatechin gallate, piceatannol, quercetin hydrate, myricetin, naringenin, and quercetin 3-β-D-galactoside (Sigma-Aldrich). Pterostilbene was purchased from TCI (Tokyo, Japan).

Individual stock solutions were prepared in methanol (400–2000 mg/L). Working solutions were prepared monthly and stored in amber vials at –20 °C. Calibration standards were prepared in H₂O (0.1% formic acid/acetonitrile (95:5, v/v)) and used within three days.

Method performance was assessed by determining the limits of detection (LOD) and quantification

(LOQ), calculated at signal-to-noise ratios of 3 and 10, respectively, under established LC-DAD-FLD conditions.

Bacterial strains and growth conditions

The strains 5w, 6F, and X₁B of *Lentilactobacillus hilgardii*, isolated from Cafayate wines in Salta, Argentina (20), were selected for their ability to produce biogenic amines (21, 22). The microorganisms were activated in a MRS broth supplemented with 15% of tomato juice (MRS-TJ) at pH 5.0. Then, each strain was adapted by routinely growing it in MRS-TJ medium supplemented with 7% ethanol (99.5% v/v). After adaptation, the cells were washed three times with a sterile saline solution and inoculated into a synthetic wine-like medium (SWLM) containing 1.7% (w/v) of YNB (Difco™ & BBL™) and (in g/L): glucose 5.0; fructose 3.0; L-malic acid 3.0; tartaric acid 4.0; citric acid 0.7; K₂SO₄ 0.1; MgSO₄ 0.025 and MnSO₄ 1.0, (pH: 4.5). The SWLM was also supplemented with 10% ethanol (99.5% v/v) and a mixture of the amino acids required by these microorganisms (9). The medium was sterilized by filtration through a 0.22 μm pore size nylon membrane.

The phenolic fraction was added to the SWLM at a concentration defined as 1X, corresponding to the phenolic concentration naturally present in the original wine from which the fraction was obtained. This concentration was selected to reproduce realistic oenological conditions and to evaluate the bacterial response under phenolic levels representative of wine.

To exclude any possible effect of residual solvent used during phenolic extraction and concentration (ethyl acetate and nitrogen), control assays were performed by adding equivalent volumes of solvent-treated blanks, prepared following the same procedure but without phenolic compounds, to the culture medium.

All bacterial viability assays were performed in triplicate, independent experiments. Results are expressed as mean values, and variability among replicates was assessed to ensure reproducibility of the observed effects.

Antibacterial Activity Assay

The antibacterial activity of the LMF was measured by the determination of growth parameters of *L. hilgardii* (5w, 6F, and X₁B) in SWLM (control medium) and in SWLM individually supplemented with the LMF of young (Y) and aged (A) Tannat wines

at different concentrations. The concentration of LMF of both wines was adjusted to the same concentration as natural wines, considering the total phenolic concentrations shown in Table 1. The bacteria were inoculated at concentrations of 10^4 and 10^7 cfu/mL and incubated for 72 h at 22°C. A control assay without the addition of LMF was also conducted. Immediately after bacterial inoculation and at the end of the incubation (72 h), bacterial viability was determined by counting viable cells on MRS-agar medium. The plates were incubated at 28°C under microaerophilic conditions.

Table 1. Total phenolic compounds from wine and low molecular weight fraction from young (LMF-Y) and aged (LMF-A) from commercial red wines Tannat.

Total Phenols Compounds [mg GAE/L]	
Wine-Y	2500 ± 87.2
Wine-A	2659.16 ± 92.1
LMF-Y	433.03 ± 11.3
LMF-A	396.40 ± 12.4

Values are expressed as mean ± standard deviation.

The change in viability between the start of the inoculum and at the end of the incubation time (A) was determined as follows (Equation (1)):

$$A [\text{Log cfu/mL}] = \text{Log} (x - x_0) \quad (1)$$

where

x= viable cell concentration at the end of incubation time

x_0 = initial viable cell concentration

Statistical analysis

The means and reproducibility of the data were calculated from three independent experiments performed in duplicate. Bacterial viability was analyzed by one-way analysis of variance. Variable means showing statistical significance were compared using Tukey's test (Minitab student R12). All statements of statistical significance are based on the 0.05 level of probability.

RESULTS

Profile phenolic

Table 1 shows that the concentration of TPC-FC is similar in both young and aged wines, and a similar behavior was also observed for the LMF

obtained from these wines. On the other hand, the TPC concentrations of LMF-Y and LMF-A represent approximately 17% and 15% of the TPC determined in the respective wines.

Table 2 shows significant differences in the concentrations of total flavonoid and non-flavonoid phenolic compounds between LMF-Y and LMF-A determined by LC-DAD-FLD. In general, the concentration of TPC-LC detected in LMF-Y was slightly higher than that detected in LMF-A. The non-flavonoid compounds detected represent 63 and 59% of total phenolic compounds in LMF-A and LMF-Y, respectively.

Table 2. Composition of the low molecular weight fraction from young (LMF-Y) and aged (LMF- A) commercial Tannat red wines. Values are expressed as mean ± standard deviation.

Non-flavonoid Phenolics [mg/L]	LMF-Y	LMF-A
Gallic acid	33.04±1.3	24.55±1.1
Syringic acid	4.24±0.8	2.87±0.6
Caftaric acid	0.96±0.3	0.41±0.3
Caffeic acid	7.07±0.9	5.67±0.9
<i>p</i> -coumaric acid	6.51±0.6	3.11±0.8
OH-Tyrosol	0.60±0.3	0.13±0.1
Tyrosol	0.95±0.6	0.70±0.3
trans-resveratrol	2.31±0.3	0.97±0.4
Piceatannol	0.43±0.1	0.33±0.1
Pterostilbene	0.28±0.1	0.19±0.1
<i>Total non-flavonoids</i>	56.39±2.6	38.9±1.9
Flavonoid Phenolics [mg/L]		
Procyanidin B1	4.04±0.3	0.55±0.4
(+)-catechin	5.69±0.6	3.27±0.9
Procyanidin B2	1.94±0.9	1.81±0.6
(-)-epicatechin	3.27±1.2	1.85±0.7
Quercetin-3-galactoside	1.80±1.1	0.70±0.2
Naringenin	0.10±0.1	0.13±0.1
Myricetin	4.34±1.5	2.27±1.0
Quercetin	2.59±1.1	1.36±0.6
(-)-epigallocatechin gallate	12.59±0.8	9.39±1.1
Astilbin	2.29±1.3	1.15±0.4
<i>Total flavonoids</i>	38.67±1.9	22.49±1.6
<i>Total phenolic compound</i>	95.06±2.1	61.40±1.9

Gallic acid was the main compound detected in both analyzed samples, showing the highest concentration in LMF-Y. Other major detected compounds were epigallocatechin gallate and caffeic acid, followed by (+)-catechin and *p*-coumaric acid. In addition, it was observed that most

compounds were present at significantly higher concentrations in LMF-Y, even reaching more than a twofold difference in the case of *p*-coumaric acid.

Antibacterial effect of a fraction of phenolic compounds

Figure 1 shows the effect of LMF from young and aged Tannat wines on the viability of the three spoilage LAB strains (*L. hilgardii* 5w, *L. hilgardii* 6F, *L. hilgardii* X₁B) in SWLM control medium and SWLM supplemented with LMF, using two inoculum sizes (low and high-initial cell concentration) after 72 h of incubation. Results showed that all studied LAB increased the viable cell concentration in SWLM, reaching a cell concentration nearly twofold in trials with the highest inoculum size.

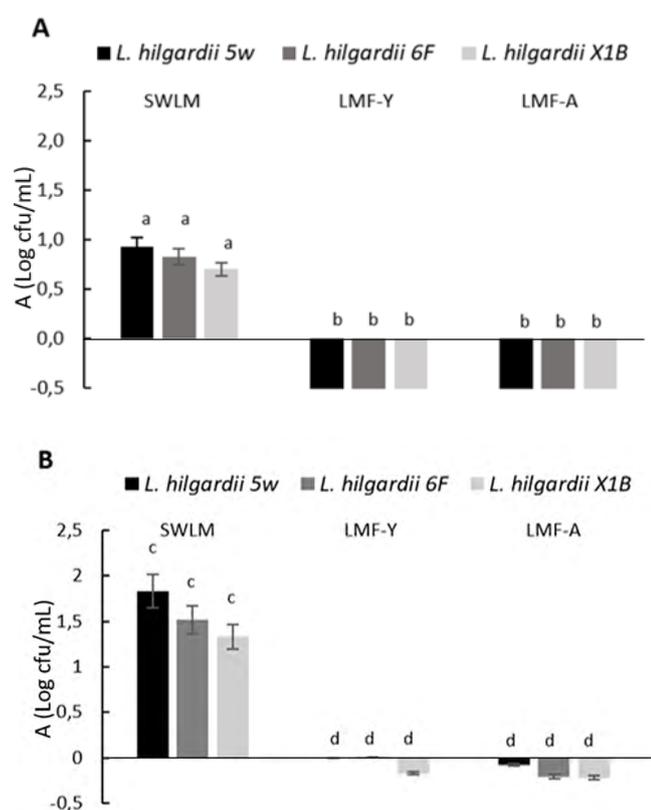


Figure 1. Effect of the low molecular weight phenolic fractions from young (LMF-Y) and aged (LMF-A) Tannat wines on the growth parameter A (Log cfu/mL) of *Lentilactobacillus hilgardii* strains (5w, 6F, and X₁B) at initial inoculum levels of 10⁴ cfu/mL (A) and 10⁷ cfu/mL (B). Values are expressed as mean ± standard deviation (n = 3). Different letters above bars indicate statistically significant differences among treatments within the same strain according to Tukey's test (p < 0.05).

In trials with low inoculum size, the presence of both assayed LMF prevented bacterial growth, also resulting in a significant loss of viability from the initial inoculum, showing negative "A" values

ranging from -0.70 and -0.98 Log cfu/mL. On the other hand, in trials conducted with a high inoculum, the presence of LMF resulted in growth arrest, with cell viability remaining close to the initial values throughout the incubation period. In general terms, the antimicrobial effect of LMF-Y and LMF-A was similar against the three *L. hilgardii* strains studied.

DISCUSSION

In the present study, we report new insights into the phenolic profile and antibacterial activity of low-molecular-weight phenolic compounds present in the LMF of young and aged wines. In general, the concentrations found of PCs in this work are in accordance with previously published literature using similar extraction procedures (7, 8,23,24,25). On the other hand, this study provides evidence that, within the same wine, the concentration of total phenolic compounds, as well as of individual low-molecular-weight compounds, decreases after aging. In effect, results from LC-DAD-FLD provide evidence that LMF-Y has a higher concentration of around 55% than LMF-A in the sum of each phenolic compound analyzed. In this way, several reports have demonstrated that the phenolic composition of wine changes during aging, reflected by modifications in color and astringency of the final product (26). These changes are mainly associated with copigmentation, cycloaddition, polymerization, and oxidation reactions involving anthocyanins and other phenolic compounds. The change in phenolic compound composition and concentration during the aging of red wines could be related to several reactions involving low-molecular-weight compounds, such as phenolic acids and flavonoids, with anthocyanins or other phenolic compounds (27,28,29), leading to the formation of more stable polymeric pigments and anthocyanin-derived compounds. In this way, Puértolas (30) reported that about 25% of flavonoid compounds may polymerize with anthocyanins at the end of the process, thereby reducing free low-molecular-weight phenolics and increasing color stability. From a technological perspective, these transformations are relevant to the wine industry, since the decrease in free low-molecular-weight phenolics during aging may influence not only antimicrobial activity but also sensory attributes such as bitterness, astringency, and overall mouthfeel. Therefore, the balance between microbial stability and organoleptic quality should be considered when evaluating the potential use of phenolic fractions as natural antimicrobial agents in wine.

Some authors report that wood compounds, such as ellagitannins, can be extracted into wine and react with flavanols, suggesting a decrease of PCs after aging (31), either through direct condensation reactions or via oxidation-mediated pathways, further modifying the phenolic profile of aged wines.

Some studies have reported that concentrations of at least 500 and 1000 mg/L of phenolic compounds are required to produce an inhibitory effect on the growth of LAB (32,6,33). Nevertheless, previous results demonstrated that 400 mg/L of protocatechuic, *trans-p*-coumaric, and *trans*-caffeic acid were enough to inhibit the growth of *L. hilgardii* and *P. pentosaceus* in wine, producing modifications in cell morphology with alterations in the microorganism cell integrity (9). Based on this previous evidence, in this study, the supplementation of SWLM with LMF was performed at 400 mg/L, showing that the LMF-Y or LMF-A produced an inhibitory effect against the three spoilage LAB assayed.

Considering that spoilage bacterial concentration usually found in wine is 10^4 cfu/mL, the decrease in bacterial viability produced by LMF supplementation in SWLM in presence of *L. hilgardii* at an inoculum size of 10^4 cfu/mL could have a great technological value. These findings suggest that low molecular weight phenolic fractions could be explored as complementary or alternative strategies to traditional antimicrobial practices in winemaking, contributing to microbial control while potentially reducing the need for chemical preservatives.

Even when a higher inoculum (10^7 cfu/mL) was utilized, both LMF-Y and LMF-A stopped the growth of all spoilage bacteria assayed. In general, LMF-Y and LMF-A showed slight differences in antimicrobial activity against the three strains studied of *L. hilgardii*. In this sense, the antimicrobial effect of LMF-Y could be sufficient to inhibit the wine-spoilage LAB, since its composition includes higher concentrations of low molecular weight PCs that are reported with more effective antimicrobial activity (6,34,7,8,9). In addition, it was reported that these compounds could exert synergistic effects between components of the fraction (7,8,34). On the other hand, individual compounds of the LMFs have previously been reported to exhibit antibacterial activity against *L. hilgardii*, *P. pentosaceus*, and other LAB species, like phenolic acids (1,3,5,6,7,8,9) flavonoids and

stilbenes (6). Nevertheless, a limitation of the present study is that the contribution of individual compounds versus synergistic interactions was not independently evaluated, and further studies would be required to elucidate the specific mechanisms involved. Overall, the results obtained in this study highlight the relevance of low-molecular-weight phenolic fractions as multifunctional components in wine, contributing not only to sensory attributes but also to microbial stability. The higher antimicrobial effectiveness observed for LMF-Y may be associated with its higher content of free phenolic acids and flavonoids. In contrast, the reduced activity in LMF-A could be related to phenolic polymerization during aging. Although the application of phenolic fractions as antimicrobial agents appears promising, further studies are required to evaluate their impact on proven organoleptic properties, optimal dosages, and possible interactions with other wine constituents under real winemaking conditions.

CONCLUSIONS

For the first time, the phenolic characterization of both young and aged Tucuman wines was performed, demonstrating that the aging process produces modifications in PCs of low molecular weight. On the other hand, the LMF obtained from young and aged wines caused growth arrest and loss of viability in spoilage lactic bacteria under the studied conditions. The slightly higher antimicrobial effectiveness observed for LMF from young wines may be related to its higher content of free phenolic acids and flavonoids, suggesting possible synergistic interactions among individual compounds.

These results suggest that LMF could represent a potential natural alternative for microbial control in winemaking, with potential application as a complementary strategy to reduce spoilage risk and enhance product stability. Nevertheless, further studies are required to assess the efficacy of the use of LMF in winemaking, including scale-up processes, evaluation under real winery conditions, the optimization of effective concentrations, and the assessment of its impact on the organoleptic properties and chemical stability of the final wine.

CONFLICT OF INTEREST

The author reports no conflict of interest.

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