







## Article

# Strategies to Reduce Sulfur Dioxide in Tannat Winemaking: Effects of Chitosan and Lysozyme on Microbial Stability, Composition, and Sensory Profile

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## Abstract

Sulfites are the most widely used additives in oenology to prevent oxidation and microbial spoilage. However, concerns about their potential adverse health effects have increased demand for low-sulfite wines. Strategies are required to ensure microbiological stability while preserving wine quality. This study evaluated strategies to reduce or replace added sulfites with chitosan and low doses of lysozyme in Tannat winemaking. It measured their effects on microbial diversity, physicochemical parameters, and sensory attributes. Treatments were vinified in triplicate: reduced sulfites (RS: 30 mg/L), chitosan (C: 100 mg/L), reduced sulfites with chitosan (RS+C: 30 mg/L + 100 mg/L), reduced sulfites with lysozyme (RS+L: 30 mg/L + 5 mg/L), and traditional winemaking (TW: 125 mg/L sulfites). Sulfur dioxide lowered lactic acid bacteria counts, whereas chitosan and lysozyme treatments maintained higher populations. Metagenomic analyses showed that sulfur dioxide reduced bacterial diversity, whereas chitosan promoted a more complex microbiota. Lysozyme selectively reduced lactic acid bacteria, mainly affecting *Oenococcus* spp. Lower sulfite levels decreased phenolic concentrations, possibly due to reduced protection against oxidation, leading to color differences among treatments. The results indicate that strategies to reduce or replace sulfites influence microbial dynamics, acidity, phenolic structure, and color, highlighting the importance of careful process management to maintain wine quality.

**Keywords:** reduced sulfur dioxide; wine microbial community; Tannat wine composition



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## 1. Introduction

Consumer demand for wines without added sulfur dioxide (SO<sub>2</sub>) has increased significantly in recent years [1]. However, producing wines under these conditions may lead to organoleptic defects or health risks due to the unchecked growth of undesirable microorganisms during alcoholic fermentation. Traditionally, SO<sub>2</sub> has been used as a preservative and antimicrobial agent in winemaking, efficiently inhibiting malolactic fermentation and microbial deterioration [2]. Nevertheless, the presence of residual sulfites in wine remains controversial due to their potential to cause adverse effects in sensitive consumers, including allergic reactions, headaches, and nausea [3,4].

Sulfur dioxide is one of the most effective additives in enology and has been used in winemaking since the 17th century. Its antiseptic, antioxidant, and enzymatic-inhibition properties make it a preferred tool for preserving wine quality [5]. Its antioxidant activity protects wines from oxidation by removing oxygen and preventing the degradation of phenolic and aromatic compounds, both essential for maintaining the wine's sensory profile [2]. Additionally, SO<sub>2</sub> inhibits the growth of yeasts, lactic acid bacteria, and acetic acid bacteria, thereby contributing to microbial stability throughout winemaking and storage [5].

Sulfur dioxide and its sulfite salts are authorized as food additives in many countries for winemaking, and the World Health Organization has established an Acceptable Daily Intake (ADI) of 0.7 mg/kg body weight per day [2]. Due to health-related issues associated with SO<sub>2</sub> consumption, the International Organization of Vine and Wine (OIV) has progressively reduced the maximum allowable concentration of SO<sub>2</sub> in wines. Excessive doses should be avoided not only for health considerations but also from an oenological perspective, as they can lead to organoleptic alterations in the final product, including aroma neutralization and the development of characteristic off-flavors [5].

The permitted level of SO<sub>2</sub> in wine has changed over time. Before 1998, there was no unified regulatory framework within the OIV, and limits varied by country. From 1998 onwards, the OIV adopted Resolution OENO 9/98, which set a maximum limit of 150 mg/L of total SO<sub>2</sub> for red wines at the time of marketing [6]. In 2018, the OIV revised certain limit values and promoted the development of technical documents and guidelines to encourage a progressive reduction in SO<sub>2</sub> content. These initiatives include recommendations for more efficient vineyard and winery management, as well as the implementation of alternative practices to minimize the necessary doses of this additive [2]. The most recent edition of the International Code of Oenological Practices (Codex OIV), particularly the 2023/2025 consolidated version, systematizes historical resolutions, including OENO 9/98, and reflects current recommendations on permitted limits and strategies for reducing the use of SO<sub>2</sub>. While technical updates have been incorporated, the numerical limits set out in Resolution OENO 9/98 remain in force [7].

Therefore, in the context of an increasingly competitive global wine market, it is essential to reduce or even eliminate the use of SO<sub>2</sub> as a preservative and to develop safer, healthier alternatives. There is growing interest in identifying alternatives or complementary agents that can reduce SO<sub>2</sub> use without compromising wine quality or safety. Among them, chitosan and lysozyme have shown promising antimicrobial properties [4].

Chitosan is a natural polysaccharide derived primarily from fungal cell walls [8]. It is considered safe for humans and has been approved for use in enology since 2009 at doses up to 100 mg/L [7]. The antimicrobial activity is mainly attributed to its polycationic nature, which disrupts the cell membranes of spoilage yeasts (*Brettanomyces* spp.) [9,10], acetic acid bacteria [11], and lactic acid bacteria [12,13], leading to cell death or growth inhibition. Studies show that chitosan can selectively inhibit *Brettanomyces* spp. growth while sparing beneficial *Saccharomyces* spp. yeast during fermentation [14]. Moreover, its application can reduce microbial activity not only during fermentation but also in wine storage, enhancing the wine's shelf life. Results reported by Paulin et al. (2020) [9] indicated that a 40 mg/L dose of chitosan effectively reduced *Brettanomyces bruxellensis* populations in most tested strains, but efficacy varied with yeast genotype and chitosan batch. On the other hand, Elmaci et al. (2015) [12] evaluated the effectiveness of chitosan against wine-related microorganisms using concentrations ranging from 200 to 2000 mg/L. They found that *Brettanomyces bruxellensis*, *Lactobacillus hilgardii*, and *Oenococcus oeni* were completely inhibited at 200 mg/L, whereas *Saccharomyces cerevisiae* showed greater resistance, remaining unaffected at these concentrations. During fermentation, chitosan

delayed the onset of fermentative activity, but did not prevent *Saccharomyces cerevisiae* from becoming viable or completing fermentation. Chitosan also exhibits antioxidant properties by chelating transition metal ions involved in oxidative reactions, thus preventing wine browning and preserving sensory attributes [8,15–17].

Lysozyme, an enzyme derived mainly from egg white, selectively targets Gram-positive bacteria, particularly lactic acid bacteria (*Pediococcus* spp., *Oenococcus* spp., and *Lactobacillus* spp.), making it useful for controlling malolactic fermentation and reducing volatile acidity [4]. Lysozyme has been approved for use in winemaking at a maximum dose of 500 mg/L and is especially effective in wines with high pH, where SO<sub>2</sub> efficacy is limited. However, lysozyme does not replace the antioxidant effect of SO<sub>2</sub> and has limited activity against Gram-negative bacteria and yeasts [18]. It can be used in combination with other antimicrobial strategies to ensure comprehensive microbial control [19]. The allergens responsible for wine allergies may include enzymes such as lysozyme; therefore, it is important to use low doses [20]. In their study, Gao et al. (2015) [18] evaluated the effectiveness of lysozyme at concentrations of 200 mg/L and 400 mg/L during alcoholic fermentation. They discovered that lysozyme effectively inhibited the growth of lactic acid bacteria responsible for spoilage without adversely affecting the fermentative activity or viability of *Saccharomyces cerevisiae*. These results suggest that lysozyme is an effective agent for controlling undesirable lactic acid bacteria during winemaking, helping to prevent sensory defects and microbiological problems.

The growing demand for sustainable viticultural practices has encouraged the adoption of strategies to reduce sulfite use in winemaking, thereby promoting more responsible production systems and improved traceability, as observed in several wine-producing countries, including Uruguay [21]. In a previous exploratory study, our research group evaluated different approaches to reduce or replace SO<sub>2</sub> during Tannat red winemaking [22]. Building on these preliminary findings, this study aims to evaluate strategies for reducing or replacing SO<sub>2</sub> in Tannat red winemaking using chitosan and lysozyme, assessing their effects on microbial composition and stability, as well as on physicochemical and sensory properties, and to provide a more comprehensive evaluation of the most effective approaches. Furthermore, the proposed strategies were designed to avoid adding substances at concentrations that could pose risks to consumer health; consequently, lysozyme was tested at doses lower than those commonly recommended.

## 2. Materials and Methods

### 2.1. Grapes and Wines

The study was conducted during the 2023 vintage using grapes of the Tannat cultivar (Vitis International Variety Catalog number VIVC 12257) [23] (*Vitis vinifera* L.). Tannat is an emblematic cultivar of Uruguay, recognized for its high productivity and oenological potential, as well as its adaptation to the country's agroclimatic conditions [24–26]. The grapes were harvested from a commercial vineyard in El Colorado, Canelones, south of Uruguay. The variety was grafted on *Vitis riparia* × *Vitis berlandieri* cv. SO4 (Selection Oppenheim 4), trained on a trellis with Guyot pruning, and managed under the Sustainable Viticulture Program [27].

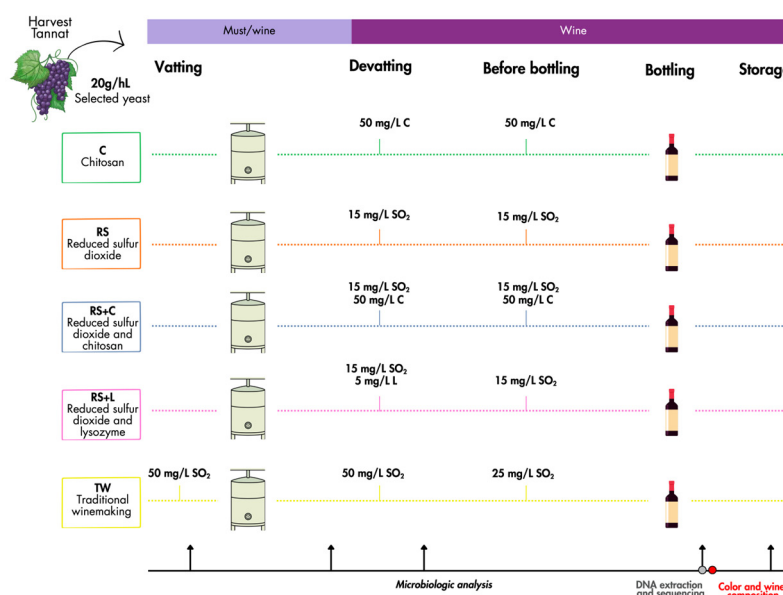
### 2.2. Harvest

When grapes reached technological maturity ( $23.0 \pm 0.8$  °Brix,  $226 \pm 9$  g sugar/L,  $3.37 \pm 0.04$  pH, and  $4.48 \pm 0.40$  g H<sub>2</sub>SO<sub>4</sub>/L of titratable acidity), 330 kg were manually harvested and transported to the Experimental Winery of the Facultad de Agronomía (Montevideo, Uruguay). The grapes were destemmed and crushed (Alfa 60 R crusher, Italcom, Piazzola Sul Brenta, Italy), and the resulting must and pomace were distributed

into 18 polyethylene containers of 10 L each. Each container received one of the reduced-sulfite treatments, sulfite replacement, or traditional winemaking.

### 2.3. Experimental Design

Eighteen containers were inoculated with selected yeast (Lamothe Abeit—Z.A. Ac-tipolis, Canéjan, France) and grouped in sets of three (Figure 1). Each group received one of the following treatments. The first treatment involved the addition of chitosan (C) at 100 mg/L (Bactiless™, Lallemand, Danstar Ferment A.G., Fredericia, Denmark). Chitosan was applied during devatting, and before bottling, the dose was divided into equal parts. The second treatment consisted of reduced sulfur dioxide (RS), in which the addition was limited to 60 mg/L of potassium metabisulfite (Kadifit, Hesse, Germany), corresponding to 30 mg/L of added sulfite. This was also applied at devatting and split into equal portions before bottling. A combined treatment of reduced sulfur dioxide and chitosan (RS+C) was applied to another group. In this case, 100 mg/L of chitosan and 60 mg/L of potassium metabisulfite were added at devatting, with the doses again divided equally before bot-tling. For the reduced sulfur dioxide and lysozyme treatment (RS+L), 60 mg/L potassium metabisulfite was added during devatting and divided before bottling, whereas 5 mg/L lysozyme (DelvoZyme, DSM, Delft, The Netherlands) was applied only during devatting. In traditional winemaking (TW), the standard dosage of potassium metabisulfite totaling 250 mg/L (equivalent to 125 mg/L of sulfur dioxide) is divided into three applications: 100 mg/L at vatting, 100 mg/L at devatting, and 50 mg/L before bottling.



**Figure 1.** Winemaking treatments and sampling timeline. C refers to chitosan, RS to reduced sulfur dioxide, RS+C to reduced sulfur dioxide and chitosan, RS+L to reduced sulfur dioxide and lysozyme, and TW to traditional winemaking. Colors denote treatments and dotted lines indicate their continuity during the process. Vertical bars indicate ad-ditive applications, and arrows mark sampling points for microbiological analysis, DNA extraction and sequencing, and wine composition analysis.

In the treatments involving sulfur dioxide reduction or replacement, a concentration of 30 mg/L SO<sub>2</sub> was applied, in accordance with the limits established by the French “Vin Méthode Nature” specification [28], which permits a maximum of 30 mg/L of added SO<sub>2</sub>. Lysozyme was applied at low doses to ensure that the reduction or replacement of sulfites did not introduce additional risks to consumer health. Although the winemaking conducted in this study does not conform to natural or minimal-intervention winemaking,

the application of comparable levels of SO<sub>2</sub> was considered appropriate to assess their effect on microbial stability and wine composition.

#### 2.4. Analytical Determinations

##### 2.4.1. Must Fermentation Kinetics

During the fermentative maceration, must density (2001FC-20/20, Alla France, Chemillé, France) and temperature (2905, Alla France, Chemillé, France) were monitored daily. The fermentation temperature was kept between 24 and 28 °C.

##### 2.4.2. Microbiological Analysis of the Fermenting Must and the Wine

Juice grape and wine samples were collected from two replicates at the following stages: vatting, before devatting, after devatting, after bottling, and during storage. Microbiological analysis of the wines was carried out in duplicate using serial decimal dilutions of the samples in peptone water by the surface plate method. Culture media and incubation conditions for each microbial group were: Plate Count Agar (PCA, Oxoid, Basingstoke, UK) for total aerobic mesophile counting (35 °C, 24–48 h), de Man Rogosa Agar (MRS, Oxoid, MRS, Oxoid) for lactic acid bacteria (37 °C, 48 h, microaerophilia), Glucose-Yeast extract Calcium Carbonate agar (GYC, Condalab, Madrid, Spain) for acetic acid bacteria (30 °C, 72 h), and Yeast extract-Peptone Dextrose agar (YPD, Neogen, Lansing, MI, USA) for yeasts (28 °C, 3–5 days). The counts were performed in triplicate and were reported as log c.f.u./mL of sample.

##### 2.4.3. DNA Extraction and Sequencing

The wine samples were extracted in two independent replicates, fifteen days after bottling. An aliquot of 30 mL of each wine sample was centrifuged for 15 min at 8000 rpm, the pellets were washed with Tris-EDTA buffer (TE) (20:2 mM) and then resuspended in 250 µL of TE [29]. Lysis and DNA extraction were performed using the ZymoBIOMICS DNA miniprep kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's protocol. The DNA was visualized on a 1% agarose gel to assess its integrity, and total DNA concentration and the 260/280 ratio were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific Inc., Wilmington, DE, USA). The genomic DNA samples were sent to Novogene Inc. (San Diego, CA, USA), where the amplicon libraries were prepared using the NovaSeq Reagent Kit (Illumina DNA PCR-Free Library Prep, San Diego, CA, USA). The hypervariable region V4 of the 16S rRNA gene from prokaryotes was obtained using primers 515F—GTGCCAGCMGCCGCGTAA and 806R—GGACTACHVGGGTWTCTAAT [30].

##### 2.4.4. Sequence Analysis

The raw sequencing data for this study have been deposited in the Sequence Read Archive (SRA) under the accession number PRJNA1367647. Raw Illumina sequencing data were processed in R (version 3.22.0) [31] using the DADA2 package (version 1.38.0) [32], following the authors' recommended workflow. After quality filtering, trimming, error learning, dereplication, and chimera removal, amplicon sequence variants (ASVs) were inferred. Taxonomic assignment was performed against the SILVA 138.1 prokaryotic SSU taxonomic training data formatted for DADA2 [32–34], using a naïve Bayesian classifier (Illumina, Inc., San Diego, CA, USA). Subsequent data handling and analyses were performed using the R packages phyloseq (version 1.54.0) [35,36] (<https://joey711.github.io/phyloseq/>, accessed on 6 August 2025) and mia (version 1.18.0; <https://github.com/microbiome/mia>, accessed on 6 August 2025). The resulting count tables were collapsed to multiple taxonomic levels (species, genus, family, and order) using functions from the mia package (version 1.18.0).

Alpha diversity was assessed after rarefaction to standardize sampling effort to the lowest sequencing depth.

#### 2.4.5. Color and Wine Composition Evaluation

The wines were analyzed in duplicate fifteen days after bottling. The ethanol content, titratable acidity, pH, malic and lactic acid contents, and volatile acidity were determined using a Winescan™ Autosampler 79,000 infrared analyzer (FOSS, Eden Prairie, MN, USA) and the Foss Integrator software version 154 (FOSS Analytical A/S, Hillerød, Denmark). The color parameters were determined directly from the wine samples placed in a 1 mm optical-path cuvette. The CIELab coordinates, lightness ( $L^*$ ), chroma ( $C^*ab$ ), hue ( $h_{ab}$ ), red-greenness ( $a^*$ ), and yellow-blueness ( $b^*$ ) were determined following the method described by Ayala et al. (1997) [37], and data processing was performed using MSCV software (version 2001; Grupo de Color, Universidad de La Rioja–Universidad de Zaragoza, Zaragoza, Spain) [38]. The concentrations of total phenols, anthocyanins, and tannins in wines were determined spectrophotometrically according to Singleton and Rossi (1965) [39], Ribéreau-Gayon and Stonestreet (1965) [40], and Sarneckis et al. (2006) [41], respectively. The HPLC separation, identification, and quantification of anthocyanins were performed on a HPLC UltiMate™ 3000 (Thermo Fisher Scientific, Germering, Germany), equipped with DAD (UltiMate® DAD-3000 Detector; Thermo Fisher Scientific, Germering, Germany), a Quaternary pump (UltiMate® LPG-3400SD; Thermo Fisher Scientific, Germering, Germany), an Autosampler (UltiMate® WPS-3000(T) SD; Thermo Fisher Scientific, Germering, Germany), and a thermostatted column compartment (UltiMate® TCC-3000SD; Thermo Fisher Scientific, Germering, Germany), and coupled to a Thermo Scientific Dionex Chro-meleon 7 Chromatography Data System Version 7.3.1. (Thermo Fisher Scientific, Germering, Germany). Wine samples (10  $\mu$ L) were injected onto a reversed-phase column (Ascentis Express C18, 2.1  $\times$  150 mm, 2.7  $\mu$ m; Sigma-Aldrich, Steinheim, Germany), and the column was thermostatted at 40 °C. The chromatographic system was based on the method previously reported by Blanco-Vega et al. (2011) [42], with minor adjustments detailed below. The mobile phase consisted of water, formic acid, and acetonitrile, prepared at 88.5:8.5:3 ( $v/v/v$ ) as solvent A and 41.5:8.5:50.0 ( $v/v/v$ ) as solvent B. The flow rate was set at 0.16 mL/min.

#### 2.4.6. Sensorial Analysis of Wines

Tannat wines were evaluated 6 months after bottling. A group of 13 assessors aged 26 to 60 years tasted the wine. Assessors were recruited among winemakers and professors from the Facultad de Agronomía at the Universidad de la República (Uruguay) and selected based on their availability to participate in the study. All the assessors had extensive previous experience in sensory evaluation of wines as part of their regular jobs. Assessors attended a 20 min training session before the evaluation to standardize the evaluation criteria. Sensory evaluation was carried out under laboratory conditions in the Oenology Laboratory of the Facultad de Agronomía. The assessors evaluated the wines blindly and in a random order. For each wine, nine sensorial attributes were evaluated on a continuous scale from 1 to 10: color intensity, hue, aromatic intensity, aromatic quality, acidity, persistence, astringency, bitterness, and preference. The values indicate the intensity of the sensation for each attribute.

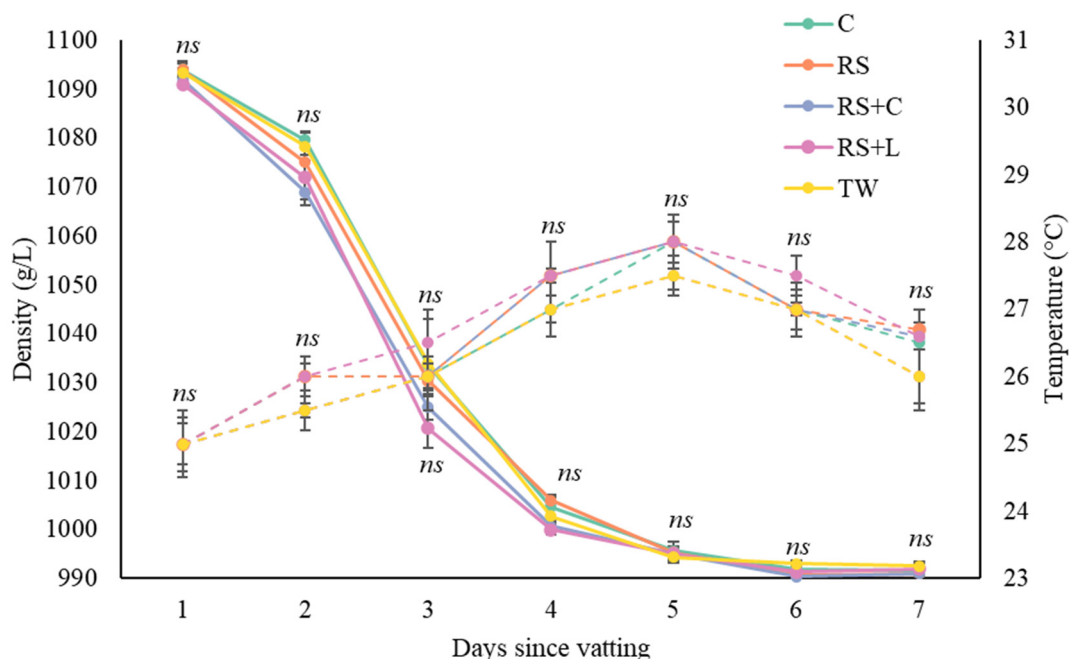
#### 2.5. Data Processing and Statistical Analysis

All data were expressed as the arithmetic average  $\pm$  standard deviation of three replicates. The data were analyzed using analysis of variance, followed by Tukey's test of means ( $\alpha \leq 0.05$ ). The programs InfoStat® v2020 (Grupo InfoStat, Universidad Nacional de Córdoba, Argentina) [43] and RStudio v4.3.2 (RStudio 2024.04.2) [31] were used for statistical analysis of the data.

### 3. Results

#### 3.1. Grape Juice Fermentation Kinetics

The evolution of density and temperature during alcoholic fermentation exhibited a similar behavior across all treatments evaluated (Figure 2). Density decreased markedly during the first six days of fermentation, reaching values below 1000 g/L from day 7, with no significant differences in fermentative kinetics among treatments. At the end of the process, all winemaking treatments stabilized at values near 995 g/L. Additionally, the temperature increased progressively during the initial days, peaking between 27 and 28 °C on days 4–6, followed by a gradual decline to approximately 26–27 °C by the end of fermentation.



**Figure 2.** Evolution of density (g/L) and temperature (°C) in Tannat must during alcoholic fermentation by treatment. Solid line: density; dashed line: temperature (same color as solid line, lighter). *ns* indicates the absence of significant differences among the treatments evaluated according to Tukey's test ( $\alpha \leq 0.05$ ). C refers to chitosan, RS to reduced sulfur dioxide, RS+C to reduced sulfur dioxide and chitosan, RS+L to reduced sulfur dioxide and lysozyme, and TW to traditional winemaking.

#### 3.2. Microbial Population Dynamics

Microbial counts varied across treatments and winemaking stages (Table 1). Total aerobic counts were initially high at the vating stage (approximately 7 log c.f.u./mL). They were similar across most treatments, except for TW, which showed significantly lower values ( $6.28 \pm 0.15$  log c.f.u./mL). However, before devatting, the total aerobic count increased slightly in the TW treatment. After devatting, viable aerobic populations markedly decreased; only the C and RS+C treatments still showed detectable values ( $5.59 \pm 0.02$  and  $2.74 \pm 0.37$  log c.f.u./mL, respectively). In contrast, counts were below the detection limit in the RS, RS+L, and TW treatments and remained below the detection limit for the remainder of the study period. The yeast population exhibited high initial loads ( $\sim 7$  log c.f.u./mL) during vating, remaining relatively stable until devatting. A strong decline occurred after devatting, particularly in treatments RS, RS+C, RS+L, and TW, whereas the C treatment maintained a significantly higher value ( $5.07 \pm 0.10$  log c.f.u./mL). After bottling, yeast populations recovered slightly in C, RS+C, and RS+L, while TW and RS showed lower counts. During storage, yeast levels increased again, reaching values between 2.56 and 3.65 log c.f.u./mL, with RS showing the lowest counts.

**Table 1.** Microbial population dynamics in Tannat wines during the vinification stages under different treatments.

| Microbial Count<br>(c.f.u./mL) | Analysis Stage   | Treatments    |               |               |               |                |
|--------------------------------|------------------|---------------|---------------|---------------|---------------|----------------|
|                                |                  | C             | RS            | RS+C          | RS+L          | TW             |
| Total aerobic count            | Vatting          | 7.11 ± 0.77 a | 7.56 ± 0.01 a | 7.11 ± 0.77 a | 7.56 ± 0.01 a | 6.28 ± 0.15 b  |
|                                | Before devatting | 7.00 ± 0.47 b | 6.67 ± 0.23 b | 7.00 ± 0.47 b | 6.67 ± 0.23 b | 7.67 ± 0.01 a  |
|                                | After devatting  | 5.59 ± 0.02 a | <1            | 2.74 ± 0.37 b | <1            | <1             |
|                                | After bottling   | <1            | <1            | <1            | <1            | <1             |
|                                | Storage          | <1            | <1            | <1            | <1            | <1             |
| Yeast                          | Vatting          | 7.24 ± 0.03 a | 7.24 ± 0.02 a | 7.24 ± 0.03 a | 7.24 ± 0.02 a | 7.32 ± 0.14 a  |
|                                | Before devatting | 7.14 ± 0.15 a | 6.92 ± 0.32 a | 7.14 ± 0.15 a | 6.92 ± 0.32 a | 7.63 ± 0.18 a  |
|                                | After devatting  | 5.07 ± 0.10 a | 1.00 ± 0.10 b | 1.00 ± 0.10 b | 1.00 ± 0.10 b | 1.00 ± 0.10 b  |
|                                | After bottling   | 1.60 ± 0.10 a | 1.00 ± 0.10 b | 1.70 ± 0.10 a | 1.66 ± 0.26 a | 1.15 ± 0.21 ab |
|                                | Storage          | 3.34 ± 0.31 a | 2.58 ± 0.25 b | 3.65 ± 0.77 a | 3.09 ± 0.73 a | 3.52 ± 0.06 a  |
| Lactic acid bacteria           | Vatting          | 6.03 ± 0.17 a | 5.70 ± 0.05 a | 6.03 ± 0.17 a | 5.70 ± 0.06 a | 6.12 ± 0.41 a  |
|                                | Before devatting | 3.80 ± 0.28 a | 4.06 ± 0.08 a | 3.80 ± 0.28 a | 4.06 ± 0.08 a | 3.00 ± 0.10 a  |
|                                | After devatting  | 2.08 ± 0.34 b | 3.31 ± 0.15 a | 2.48 ± 0.10 b | 2.48 ± 0.10 b | 1.59 ± 0.16 b  |
|                                | After bottling   | 3.37 ± 0.04 b | 4.48 ± 0.10 a | 1.95 ± 0.10 c | 1.70 ± 0.10 c | 1.30 ± 0.43 c  |
|                                | Storage          | 2.58 ± 0.37 b | 2.25 ± 0.26 b | 3.07 ± 0.71 a | 3.01 ± 0.64 a | -              |
| Acetic acid bacteria           | Vatting          | 3.72 ± 0.36 a | 3.72 ± 0.36 a | 3.72 ± 0.36 a | 3.72 ± 0.36 a | 4.06 ± 0.27 a  |
|                                | Before devatting | <1            | <1            | <1            | <1            | <1             |
|                                | After devatting  | <1            | <1            | <1            | <1            | <1             |
|                                | After bottling   | <1            | <1            | <1            | <1            | <1             |
|                                | Storage          | <1            | <1            | <1            | <1            | <1             |

Means with different letters indicate significant differences among the evaluated treatments according to Tukey’s test ( $\alpha \leq 0.05$ ). Values reported as “<1 log c.f.u./mL” indicate counts below the limit of detection (LOD) of the plating method. The assay’s minimum detection limit was 1 log c.f.u./mL; all replicate samples were below this threshold, with no colonies observed. C refers to chitosan, RS to reduced sulfur dioxide, RS+C to reduced sulfur dioxide and chitosan, RS+L to reduced sulfur dioxide and lysozyme, and TW to traditional winemaking.

Lactic acid bacteria (LAB) were initially present at moderate levels (5.7–6.1 log c.f.u./mL) during vatting. Counts decreased before devatting and remained low after devatting across all treatments. After bottling, C and RS wines exhibited the highest LAB counts, with counts increasing relative to the previous sampling time. By contrast, other treatments continued to decline. During storage, LAB counts were below the detection limits for the TW wines, whereas in the RS+C and RS+L treatments, LAB increased slightly (~3 log c.f.u./mL). Acetic acid bacteria (AAB) were detected only during vatting, with comparable counts across treatments (3.7–4.1 log c.f.u./mL), and were not recovered in subsequent stages.

### 3.3. Diversity of Microbial Community

In this study, we assessed and compared the microbial communities across different winemaking treatments at 15 days post-bottling using 16S rRNA gene sequencing. Hence, the results provide a ‘snapshot’ of the bacterial microbiota after 15 days of storage. Deep sequencing of microbial communities generated a total of 1,494,432 V4-region sequences from C, RS, RS+C, RS+L, and TW. After quality filtering, trimming, error learning, dereplication, and chimera removal, a total of 2029 V4 amplicon sequence variants (ASVs) were inferred. To assess variation in microbial biodiversity, the Chao1 richness estimator and Shannon diversity index were used to compare all treatments (Table 2). The sequencing results revealed marked differences in bacterial diversity and richness among the Tannat wines. The total number of raw reads was similar across samples, whereas the numbers of ASVs and genera detected varied considerably between treatments. Although results should be interpreted with caution, as differences were observed between duplicates in some treatments, the high genus-level assignment percentage (>99% in most samples) supports high

sequencing quality and reliable taxonomic resolution. The traditional winemaking sample (TW) exhibited moderate richness and diversity (Chao1: 183; Shannon: 2.0), indicating a moderate level of microbial complexity relative to the other treatments (Table 2). The RS treatment exhibited the lowest diversity and richness, suggesting strong dominance by a few bacterial taxa or by less diverse communities. In contrast, the RS+C treatment presented the highest values for both Shannon and Chao1 indices, indicating a complex and diverse bacterial community structure (Table 2). Additionally, the rarefaction curves in all cases reached a sufficient sequencing depth to capture the full diversity present in the samples, indicating that the sequencing effort was adequate and that the microbial communities were comprehensively represented.

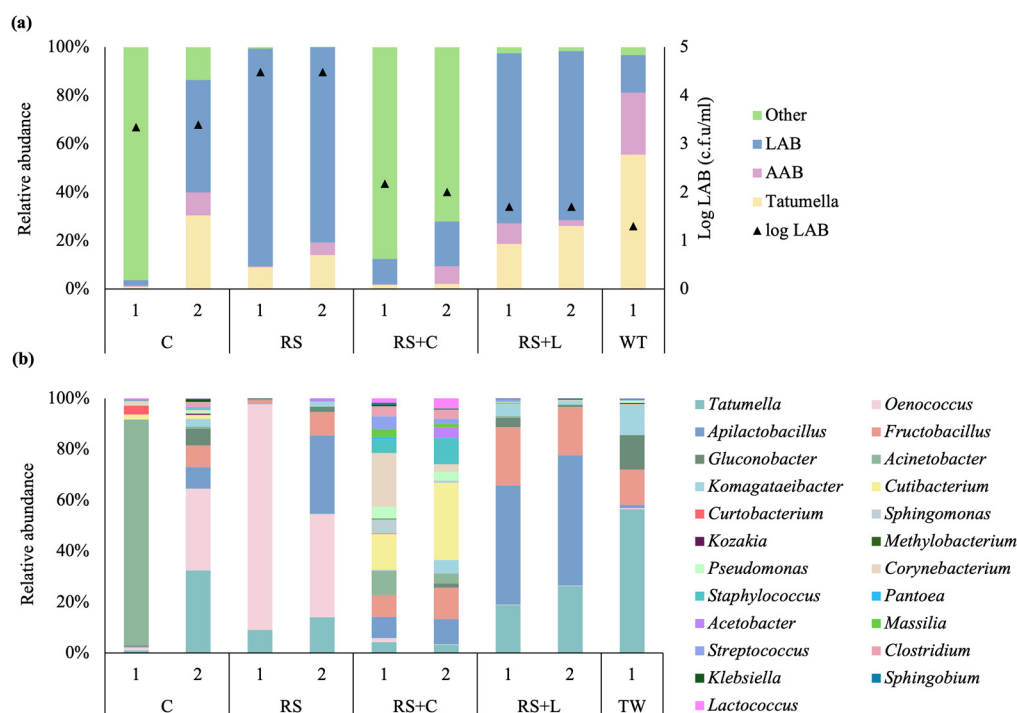
**Table 2.** Summary of Illumina sequencing data, ASV richness, sample coverage, and indices (Shannon and Chao1) for bacterial 16S rDNA libraries from Tannat wines.

|                                 | Treatments |         |         |         |         |         |         |         |         |  |
|---------------------------------|------------|---------|---------|---------|---------|---------|---------|---------|---------|--|
|                                 | C          |         | RS      |         | RS+C    |         | RS+L    |         | TW      |  |
|                                 | 1          | 2       | 1       | 2       | 1       | 2       | 1       | 2       | 1       |  |
| Raw reads                       | 109,816    | 179,059 | 183,538 | 175,799 | 180,056 | 184,566 | 130,254 | 185,791 | 165,553 |  |
| ASVs                            | 94         | 294     | 132     | 37      | 608     | 351     | 165     | 167     | 181     |  |
| % reads assigned at genus level | 0.995      | 0.993   | 0.999   | 1       | 0.893   | 0.938   | 0.999   | 0.997   | 0.997   |  |
| Number of genus                 | 54         | 124     | 73      | 15      | 233     | 141     | 107     | 103     | 102     |  |
| Shannon diversity index         | 1.59       | 2.58    | 0.59    | 1.64    | 5.25    | 4.37    | 1.73    | 1.52    | 2.00    |  |
| Chao1 richness index            | 94         | 294     | 133     | 36      | 611     | 352     | 166     | 164     | 183     |  |

Columns represent a replicate (1 and 2) sample obtained from each treatment (C, RS, RS+L, RS+C) and traditional winemaking wine (TW).

The dynamics of the bacterial population in all wines are shown in Figure 3. Two different repetitions for each treatment are represented. Replicates were comparable across all samples, except for the C treatment, in which replicate 1 showed a high abundance of non-oenological aerobic bacteria, *Acinetobacter johnsonii* (84%) (Figure 3a,b). For all treatments except RS+C, the taxonomic composition of the bacterial microbiota could be summarized as *Tatumella* spp., acetic acid bacteria (AAB), and lactic acid bacteria (LAB). Nevertheless, the relative abundances of these groups varied among treatments (Figure 3a). The genus *Tatumella* spp. (phylum Proteobacteria) was the most abundant bacterial taxon in TW, accounting for 55.6% of the relative abundance. While the relative abundance of *Tatumella* spp. was higher in TW than in other winemaking strategies, it was present in all treatments, with relative abundances ranging from 1.8 to 30.4%. For our analysis, all ASVs from genera typically assigned to AAB (i.e., *Gluconobacter*, *Acetobacter*) and LAB (i.e., *Oenococcus*, *Lactobacillus*) were combined (Figure 3a). AAB relative abundance ranged from less than 1% in RS1 to 25% in TW. The AAB population was higher in TW than in other winemaking strategies (Figure 3a). *Gluconobacter oxydans* and *Komagataeibacter saccharivorans* were the most abundant species in TW. These species were the most abundant AAB in all winemaking strategies (Figure 3b). Among LAB populations, this group was the most abundant in the C2, RS, and RS+L treatments, with relative abundances ranging from 46 to 89% (Figure 3a). This dominance was not evident in RS+C and TW wines, with relative abundances of approximately 15%. Considering the taxonomic composition of LAB communities, differences at the species level were observed between treatments. Overall, the most abundant genera were *Oenococcus*, *Fructobacillus*, and *Apilactobacillus*. Due to its tolerance of winemaking conditions, *Oenococcus oeni* is the predominant lactic acid bacterium in wines and plays a crucial role in malolactic fermentation. According to the results presented in Figure 3b, *Oenococcus oeni* was the most represented LAB species in C2 and RS wines. On the contrary, the relative abundance of *Oenococcus oeni* was less than 1% in

RS+L, RS+C, and TW wines. *Fructobacillus* spp. was detected for all treatments but was the predominant LAB genus only in traditional vinification (Figure 3b). *Apilactobacillus* spp. was predominant in samples in which *Oenococcus* spp. was not the dominant genus, as observed for the RS+L treatment (Figure 3b). Despite differences in LAB community composition, winemaking in which *Oenococcus oeni* predominated showed the highest LAB counts (Figure 3a). RS+C winemaking was the only treatment in which bacterial composition could not be explained by *Tatumella* spp., AAB, and LAB genera. ASVs recovered were 608 and 351 for RS+C1 and RS+C2, respectively. *Tatumella* spp., AAB, and LAB genera accounted for 1.9%, 7.3%, and 14.5% of the bacterial population, respectively. The other 76% comprised diverse genera, including *Cutibacterium*, *Corynebacterium*, *Staphylococcus*, *Pseudomonas*, *Clostridium*, and *Sphingomonas*. It is worth noting that the LAB group was more diverse in these samples, including genera such as *Lactococcus* and *Streptococcus* (Figure 3b).



**Figure 3.** Relative abundances of the bacterial community for Tannat wines after the bottling stage. Columns represent a replicate (1 and 2) sample obtained from each treatment (C, RS, RS+L, RS+C) and traditional winemaking wine (TW). The most abundant bacterial groups are shown in (a) and at the genus level (b). The category “Other” includes taxa that were either unclassified at the genus level or classified genera with very low relative abundance.

### 3.4. General Composition of Tannat Wines

Strategies aimed at replacing or reducing added sulfite content modified the basic composition of wines (Table 3). In terms of ethanol content, the RS, RS+L, and RS+C treatments showed the lowest levels (12.6–12.7% *v/v*), which were significantly lower than those observed in the C treatment (13.1 ± 0.2% *v/v*). TW wines showed intermediate ethanol levels, with no significant differences compared to the other treatments.

Slight variations in pH were observed between the different treatments. The RS (3.73 ± 0.03) and RS+C (3.76 ± 0.02) treatments had significantly lower pH values than the control (3.88 ± 0.02). By contrast, the highest pH values were observed in C wines (3.89 ± 0.01).

Titrateable acidity ranged from 3.57 to 4.32 g/L of H<sub>2</sub>SO<sub>4</sub>. The highest value (4.32 ± 0.79 g/L) was observed in RS+C wines. TW wines had the highest malic acid con-

tent ( $2.30 \pm 0.09$  g/L) and the lowest lactic acid content ( $1.18 \pm 0.17$  g/L). RS+C and RS+L wines exhibited distinct acid profiles, with the lowest malic acid content (1.42–1.73 g/L) and the highest lactic acid content (2.23–2.50 g/L). Volatile acidity remained low across all treatments (0.33–0.47 g/L) and did not differ significantly.

**Table 3.** General analytic parameters of Tannat wines by treatment.

| Analytical Parameter      | Treatments                |                           |                           |                          |                          |
|---------------------------|---------------------------|---------------------------|---------------------------|--------------------------|--------------------------|
|                           | C                         | RS                        | RS+C                      | RS+L                     | TW                       |
| Ethanol (% <i>v/v</i> )   | $13.1 \pm 0.2$ <i>a</i>   | $12.6 \pm 0.1$ <i>b</i>   | $12.7 \pm 0.3$ <i>b</i>   | $12.6 \pm 0.1$ <i>b</i>  | $12.8 \pm 0.2$ <i>ab</i> |
| pH                        | $3.89 \pm 0.01$ <i>a</i>  | $3.73 \pm 0.03$ <i>c</i>  | $3.76 \pm 0.02$ <i>c</i>  | $3.81 \pm 0.03$ <i>b</i> | $3.85 \pm 0.02$ <i>b</i> |
| Titrateable acidity (g/L) | $3.57 \pm 0.08$ <i>b</i>  | $3.97 \pm 0.12$ <i>ab</i> | $4.32 \pm 0.79$ <i>a</i>  | $3.62 \pm 0.16$ <i>b</i> | $3.57 \pm 0.08$ <i>b</i> |
| Malic acid (g/L)          | $1.53 \pm 0.05$ <i>cd</i> | $1.97 \pm 0.08$ <i>b</i>  | $1.73 \pm 0.34$ <i>bc</i> | $1.42 \pm 0.12$ <i>d</i> | $2.30 \pm 0.09$ <i>a</i> |
| Lactic acid (g/L)         | $1.78 \pm 0.04$ <i>b</i>  | $1.58 \pm 0.10$ <i>b</i>  | $2.50 \pm 0.43$ <i>a</i>  | $2.23 \pm 0.08$ <i>a</i> | $1.18 \pm 0.17$ <i>c</i> |
| Volatile acidity (g/L)    | $0.43 \pm 0.02$ <i>a</i>  | $0.44 \pm 0.07$ <i>a</i>  | $0.47 \pm 0.10$ <i>a</i>  | $0.46 \pm 0.04$ <i>a</i> | $0.33 \pm 0.04$ <i>a</i> |
| Fructose (g/L)            | $0.95 \pm 0.14$ <i>a</i>  | $0.98 \pm 0.08$ <i>a</i>  | $0.82 \pm 0.19$ <i>a</i>  | $0.90 \pm 0.14$ <i>a</i> | $0.97 \pm 0.10$ <i>a</i> |
| Glucose (g/L)             | $1.80 \pm 0.11$ <i>d</i>  | $2.32 \pm 0.12$ <i>c</i>  | $3.20 \pm 0.23$ <i>a</i>  | $2.77 \pm 0.19$ <i>b</i> | $1.57 \pm 0.08$ <i>d</i> |
| Glucose/Fructose ratio    | $2.40 \pm 0.18$ <i>a</i>  | $2.17 \pm 0.16$ <i>a</i>  | $1.25 \pm 0.10$ <i>c</i>  | $1.72 \pm 0.38$ <i>b</i> | $2.27 \pm 0.18$ <i>a</i> |

Means with different letters indicate significant differences among the evaluated treatments according to Tukey's test ( $\alpha \leq 0.05$ ). C refers to chitosan, RS to reduced sulfur dioxide, RS+C to reduced sulfur dioxide and chitosan, RS+L to reduced sulfur dioxide and lysozyme, and TW to traditional winemaking. Titrateable acidity is expressed in g of H<sub>2</sub>SO<sub>4</sub> per liter, and volatile acidity is expressed in g of acetic acid per liter.

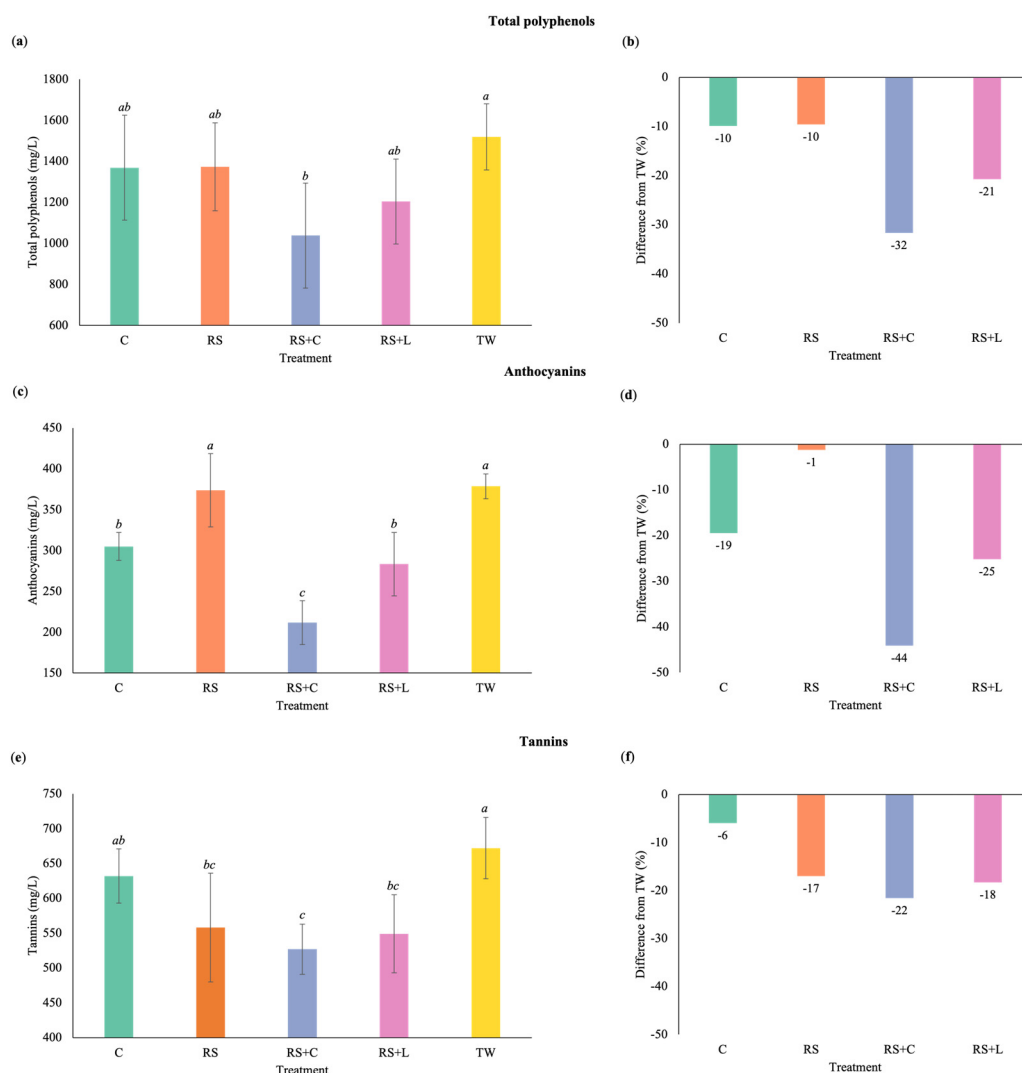
Regarding residual sugars, fructose concentrations were low and comparable across treatments (0.82–0.98 g/L), indicating that alcoholic fermentation was largely complete. However, glucose concentrations showed variations, with higher levels observed in the RS+C treatment ( $3.20 \pm 0.23$  g/L). This trend is reflected in the glucose-to-fructose ratio, which was lower in the RS+C treatment ( $1.25 \pm 0.10$ ), whereas higher ratios were observed in RS, TW, and C wines ( $2.17 \pm 0.16$ – $2.40 \pm 0.18$ ).

### 3.5. Phenolic Composition of Tannat Wines

The evaluated treatments significantly affected the phenolic composition of the wines (Figure 4). Total phenolic content was highest in TW wines ( $1519 \pm 161$  mg/L), whereas lower concentrations were observed in the remaining treatments. Wines subjected to the RS+C treatment exhibited the lowest total polyphenol content, a 32% reduction relative to TW wines. Anthocyanin concentrations were highest in RS and TW wines ( $374 \pm 45$  and  $379 \pm 15$  mg/L, respectively). In contrast, RS+L and C wines showed approximately 20% lower anthocyanin levels ( $283 \pm 39$  and  $305 \pm 17$  mg/L, respectively). The lowest anthocyanin concentration was detected in RS+C wines ( $212 \pm 27$  mg/L), corresponding to a 44% decrease relative to TW wines. Tannin content was also significantly influenced by the applied treatments. TW wines presented the highest tannin concentration ( $672 \pm 44$  mg/L), while all other treatments exhibited lower values. The RS+C treatment resulted in the lowest tannin concentration ( $527 \pm 36$  mg/L), which was 22% lower than that observed in TW wines.

The anthocyanin profile of the wines (Table 4) differed among the treatments. The RS+C wines had the highest non-acylated anthocyanins ( $83.8 \pm 0.5$ ), while RS wines had the lowest proportion ( $80.6 \pm 1.1$ ). However, the proportions in C and RS+L wines ( $82.8 \pm 0.2$  and  $82.3 \pm 0.9$ , respectively) were comparable to those in TW ( $80.9 \pm 0.1$ ). When expressed relative to TW (Table 5), most treatments showed reductions in acylated fractions, particularly RS+C, which presented decreases of 61% and 96% in acetylated and coumaroylated anthocyanins, respectively. In contrast, RS wines showed a 66% increase in coumaroylated anthocyanins compared to the control. Regarding the proportions of acetylated anthocyanins, RS wines had the highest value ( $19.4 \pm 0.9$ ), although there were

no significant differences between TW and RS. The other treatments showed intermediate results. RS wines also showed the highest concentration of coumaroylated anthocyanins ( $0.61 \pm 0.25$ ), similar to TW wines ( $0.37 \pm 0.04$ ), while RS+C wines had the lowest proportion ( $0.03 \pm 0.03$ ).



**Figure 4.** Phenolic compounds of Tannat wines by treatment and their differences relative to TW wines. (a) Total polyphenols by treatment; (b) Differences in total polyphenols from TW wines; (c) Anthocyanins by treatment; (d) Differences in anthocyanins from TW wines; (e) Tannins by treatment; (f) Differences in tannins from TW wines. Means with different letters indicate significant differences among the evaluated treatments according to Tukey’s test ( $\alpha \leq 0.05$ ). C refers to chitosan, RS to reduced sulfur dioxide, RS+C to reduced sulfur dioxide and chitosan, RS+L to reduced sulfur dioxide and lysozyme, and TW to traditional winemaking.

Of the most important anthocyanins found in wine, malvidin was the most abundant in all treatments, followed by petunidin. TW wines had the highest proportions of all anthocyanins, except for malvidin. RS+C and RS+L wines showed the highest proportions of malvidin ( $93.4 \pm 1.1$  and  $92.6 \pm 1.6$ , respectively), whereas TW and RS presented lower values ( $87.9 \pm 0.3$  and  $87.3 \pm 1.3$ ). In contrast, other pigments, such as delphinidin, petunidin, and peonidin, tended to decrease in the alternative treatments relative to TW, with relative reductions of up to 82%, 68%, and 87%, respectively (Table 5).

**Table 4.** Anthocyanin profile of Tannat wines by treatment.

| Anthocyanins Profile (%)   | Treatments            |                      |                      |                       |                       |
|----------------------------|-----------------------|----------------------|----------------------|-----------------------|-----------------------|
|                            | C                     | RS                   | RS+C                 | RS+L                  | TW                    |
| Non-acylated anthocyanins  | 82.8 ± 0.2 <i>ab</i>  | 80.6 ± 1.1 <i>d</i>  | 83.8 ± 0.5 <i>a</i>  | 82.3 ± 0.9 <i>abc</i> | 80.9 ± 0.1 <i>cd</i>  |
| Acetylated anthocyanins    | 17.0 ± 0.2 <i>cd</i>  | 19.4 ± 0.9 <i>a</i>  | 16.2 ± 0.5 <i>d</i>  | 17.6 ± 0.9 <i>bcd</i> | 18.7 ± 0.1 <i>ab</i>  |
| Coumaroylated anthocyanins | 0.19 ± 0.01 <i>bc</i> | 0.61 ± 0.25 <i>a</i> | 0.03 ± 0.03 <i>c</i> | 0.11 ± 0.13 <i>bc</i> | 0.37 ± 0.04 <i>ab</i> |
| Delphinidin                | 2.20 ± 0.17 <i>ab</i> | 3.00 ± 0.47 <i>a</i> | 0.95 ± 0.24 <i>c</i> | 1.27 ± 0.60 <i>bc</i> | 2.50 ± 0.11 <i>a</i>  |
| Cyanidin                   | -                     | -                    | -                    | -                     | -                     |
| Petunidin                  | 7.00 ± 0.29 <i>a</i>  | 7.79 ± 0.63 <i>a</i> | 4.89 ± 0.33 <i>b</i> | 5.35 ± 0.67 <i>b</i>  | 6.85 ± 0.20 <i>a</i>  |
| Peonidin                   | 1.06 ± 0.11 <i>bc</i> | 1.90 ± 0.33 <i>b</i> | 0.73 ± 0.50 <i>c</i> | 0.80 ± 0.32 <i>c</i>  | 2.76 ± 0.14 <i>a</i>  |
| Malvidin                   | 89.7 ± 0.6 <i>bc</i>  | 87.3 ± 1.3 <i>c</i>  | 93.4 ± 1.1 <i>a</i>  | 92.6 ± 1.6 <i>a</i>   | 87.9 ± 0.3 <i>c</i>   |

Means with different letters indicate significant differences among the evaluated treatments according to Tukey’s test ( $\alpha \leq 0.05$ ). C refers to chitosan, RS to reduced sulfur dioxide, RS+C to reduced sulfur dioxide and chitosan, RS+L to reduced sulfur dioxide and lysozyme, and TW to traditional winemaking.

**Table 5.** Differences in anthocyanin profile from TW wines.

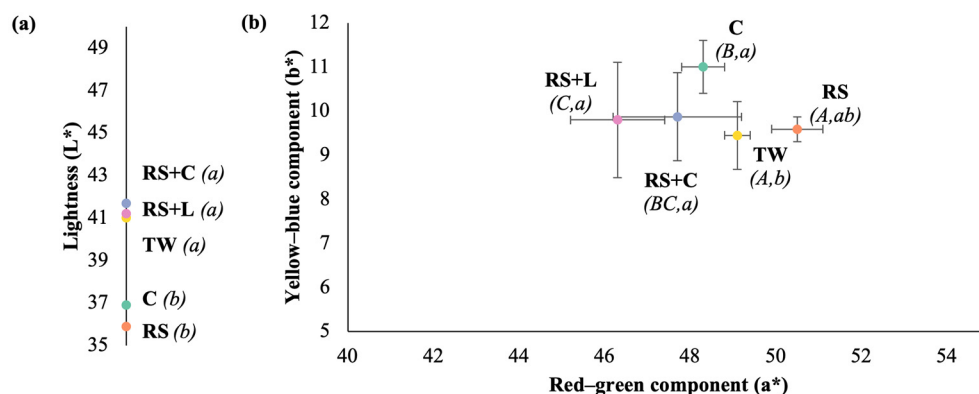
| Anthocyanins Profile (%)   | Difference from TW (%) |     |      |      |     |
|----------------------------|------------------------|-----|------|------|-----|
|                            | C                      | RS  | RS+C | RS+L | TW  |
| Non-acylated anthocyanins  | -40                    | -3  | -54  | -48  | -40 |
| Acetylated anthocyanins    | -47                    | 2   | -61  | -52  | -47 |
| Coumaroylated anthocyanins | -71                    | 66  | -96  | -82  | -71 |
| Delphinidin                | -48                    | 20  | -82  | -72  | -48 |
| Cyanidin                   | -                      | -   | -    | -    | -   |
| Petunidin                  | -40                    | 12  | -68  | -59  | -40 |
| Peonidin                   | -77                    | -31 | -87  | -84  | -77 |
| Malvidin                   | -40                    | -2  | -52  | -46  | -40 |

C refers to chitosan, RS to reduced sulfur dioxide, RS+C to reduced sulfur dioxide and chitosan, RS+L to reduced sulfur dioxide and lysozyme, and TW to traditional winemaking.

### 3.6. Color Parameters of Tannat Wines

The colorimetric evaluation based on CIELAB parameters ( $L^*$ ,  $a^*$ ,  $b^*$ ,  $C^*ab$  and  $h_{ab}$ ) revealed significant differences in the visual characteristics of the wines depending on the applied treatment (Figure 5 and Table 6). C and RS wines exhibited the lowest  $L^*$  values ( $36.9 \pm 0.5$  and  $35.9 \pm 2.6$ , respectively), corresponding to a 10–13% decrease compared with TW wines (Table 7). In contrast, TW, RS+L, and RS+C wines showed higher  $L^*$  values, indicating a lighter color. Consistently, the relative comparison with TW showed minimal changes in lightness for RS+L (0%) and RS+C (+1%), confirming that these treatments preserved brightness values close to the control (Table 7). All samples displayed positive  $a^*$  and  $b^*$  coordinates, confirming a chromatic profile dominated by red and yellow hues, respectively. RS wines exhibited the highest  $a^*$  value, indicating a more intense red coloration, although this value differed by 3% from that of TW wines. Regarding the  $b^*$  coordinate, C, RS+L, and RS+C wines exhibited the highest values, suggesting a more pronounced yellow hue. Particularly, C wines showed a  $b^*$  value 16% higher than that of TW wines. Conversely, TW wines presented lower  $b^*$  values, followed by RS wines.

Chroma ( $C^*ab$ ) and hue angle ( $h_{ab}$ ) differed significantly among the evaluated treatments (Table 6). RS wines exhibited the highest chroma value ( $51.4 \pm 0.6$ ), approximately 3% higher than that observed in TW wines (Table 7), indicating a more vivid, saturated color. This was followed by TW ( $50.0 \pm 0.4$ ) and C wines ( $49.5 \pm 0.5$ ), which did not differ significantly. In contrast, RS+L wines exhibited the lowest chroma value ( $47.3 \pm 1.4$ ), representing a 5% decrease relative to TW wines. Table 7 shows small reductions in chroma for C (−1%) and RS+C (−3%), confirming a slight decrease in color saturation in these treatments.



**Figure 5.** Lightness (a) and distribution of Tannat wines in the CIELAB a\*-b\* plane (b) by treatment. Means with lowercase letters indicate significant differences in lightness (L\*) and the yellow–blue component (b\*) according to Tukey’s test ( $\alpha \leq 0.05$ ). Means with uppercase letters indicate significant differences in the red–green component (a\*) according to Tukey’s test ( $\alpha \leq 0.05$ ). C refers to chitosan, RS to reduced sulfur dioxide, RS+C to reduced sulfur dioxide and chitosan, RS+L to reduced sulfur dioxide and lysozyme, and TW to traditional winemaking.

**Table 6.** Chroma (C\*ab) and Hue (h ab) in Tannat wines by treatment.

| Color Parameter | Treatments   |              |               |               |               |
|-----------------|--------------|--------------|---------------|---------------|---------------|
|                 | C            | RS           | RS+C          | RS+L          | TW            |
| C*ab            | 49.5 ± 0.5 b | 51.4 ± 0.6 a | 48.7 ± 1.7 bc | 47.3 ± 1.4 c  | 50.0 ± 0.4 ab |
| h ab            | 12.9 ± 0.6 a | 10.8 ± 0.3 b | 11.7 ± 0.9 ab | 11.9 ± 1.3 ab | 10.9 ± 0.8 b  |

Means with different letters indicate significant differences among the evaluated treatments according to Tukey’s test ( $\alpha \leq 0.05$ ). C refers to chitosan, RS to reduced sulfur dioxide, RS+C to reduced sulfur dioxide and chitosan, RS+L to reduced sulfur dioxide and lysozyme, and TW to traditional winemaking.

**Table 7.** Differences in color composition from TW wines.

| Color Parameter | Difference from TW (%) |     |      |      |
|-----------------|------------------------|-----|------|------|
|                 | C                      | RS  | RS+C | RS+L |
| L*              | −10                    | −13 | 1    | 0    |
| a*              | −2                     | 3   | −3   | −6   |
| b*              | 16                     | 1   | 4    | 4    |
| C*ab            | −1                     | 3   | −3   | −5   |
| h ab            | 18                     | −1  | 7    | 9    |

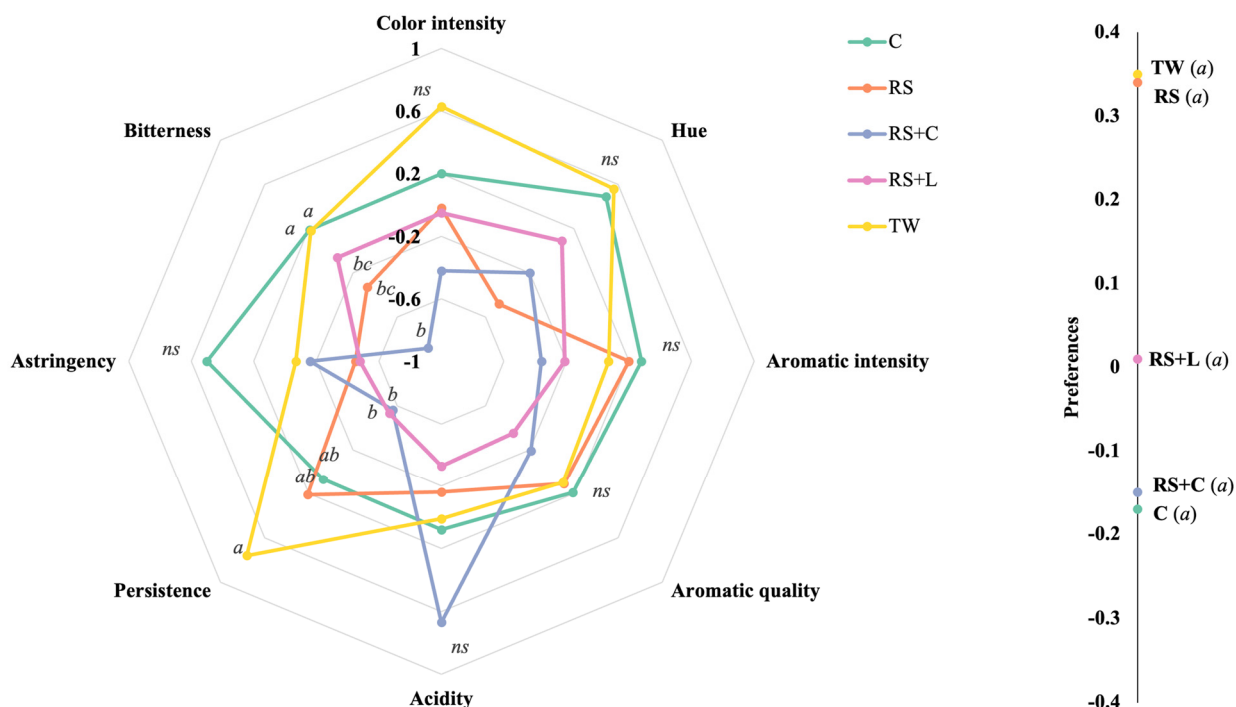
C refers to chitosan, RS to reduced sulfur dioxide, RS+C to reduced sulfur dioxide and chitosan, RS+L to reduced sulfur dioxide and lysozyme, and TW to traditional winemaking.

Regarding hue angle (h ab), values ranged from 10.8 to 12.9, reflecting differences in perceived color shade. C wines exhibited the highest hue angle (12.9 ± 0.6), corresponding to an 18% increase relative to TW wines. The remaining treatments exhibited lower hue angles, with no statistically significant differences among them. Compared with TW, RS+C and RS+L showed moderate increases in hue angle (+7% and +9%, respectively), whereas RS showed no change relative to the control (−1%), indicating minor shifts in tonality among treatments.

### 3.7. Sensory Attributes of Tannat Wines

The sensory descriptive analysis revealed significant differences in several organoleptic attributes among the evaluated wine treatments (Figure 6). Statistical analysis indicated significant differences ( $p < 0.05$ ) among treatments for bitterness and persistence. No significant differences were observed in color intensity, hue, aromatic intensity, aromatic

quality, astringency, or acidity, indicating that these characteristics were perceived similarly across all treatments by the assessors.



**Figure 6.** Sensory attributes. Means with different letters indicate significant differences in persistence and bitterness according to Tukey’s test ( $\alpha \leq 0.05$ ). *ns* indicates the absence of significant differences among the treatments evaluated according to Tukey’s test ( $\alpha \leq 0.05$ ). C refers to chitosan, RS to reduced sulfur dioxide, RS+C to reduced sulfur dioxide and chitosan, RS+L to reduced sulfur dioxide and lysozyme, and TW to traditional winemaking.

TW wines consistently showed the highest color intensity and hue. C wines also achieved elevated values for color-related attributes and aromatic intensity and quality, comparable to TW.

Regarding wine persistence, TW wines showed the highest score, suggesting a longer-lasting flavor perception. RS+C and RS+L wines showed intermediate values across most attributes. However, RS+C exhibited the highest acidity, with no significant differences among the other treatments.

The sensory preference evaluation showed no statistically significant differences among the treatments (Figure 6). Mean preference scores were slightly higher for the TW and RS wines, while C and RS+C presented the lowest mean values.

#### 4. Discussion

The evolution of density and temperature indicated that alcoholic fermentation proceeded rapidly and was completed in all treatments, with no statistically significant differences observed. This suggests that the selected yeast *Saccharomyces* spp. inoculated colonizes the must efficiently without altering fermentation kinetics, even in treatments C, RS, RS+C, and RS+L, where no additives were added before fermentation. The SO<sub>2</sub> reduction or substitution strategies evaluated did not affect the completion of fermentation or the production of ethanol on a practical scale.

Microbial counts varied with the treatment and winemaking stage. The reduction in microbial populations after devatting and bottling in RS, RS+L, and TW wines suggests that SO<sub>2</sub> has a selective effect on the survival of susceptible microorganisms, regardless of the dose used [44]. In contrast, treatment with chitosan (C) maintained higher counts of

yeasts and LAB at certain stages, suggesting that the fractional application of 100 mg/L was not fully effective in suppressing these microbial populations across all winemaking stages. This is consistent with reports from other studies, which observed heterogeneous responses of microbial groups to chitosan across species, strains, and doses [45]. Chitosan may be effective against certain microorganisms, such as *Brettanomyces* spp. and some LAB, but its efficacy varies with product concentration and origin [46]. Similarly, the reduction in counts observed in RS+L compared to LAB supports the activity of lysozyme on Gram-positive bacteria. However, the quantitative effects depend on the dose and the initial microbial composition [47,48].

The dynamics of bacterial communities across vinification treatments indicate that SO<sub>2</sub> reduction and antimicrobial strategies, such as lysozyme and chitosan, significantly affected microbial composition. The dominance of *Tatumella* spp., AAB, and LAB across most treatments supports previous findings that these taxa are major constituents of the bacterial microbiota in wines [49,50].

The predominance of *Tatumella* spp. across almost all treatments is consistent with its known association with must and early-fermentation-stage treatments, particularly under conditions that favor Proteobacteria [51,52]. A high abundance of AAB was observed in the TW and C2 treatments, primarily comprising *Gluconobacter* and *Komagataeibacter*. These bacteria can influence wine quality by producing acetic acid and other volatile compounds that contribute to spoilage [53]. However, the counts of these microorganisms were below the detection limits of the method and did not affect the physicochemical or sensory characteristics of the wines.

Conversely, treatments such as RS and RS+L showed a clear dominance of LAB, particularly *Oenococcus oeni*, *Fructobacillus*, and *Apilactobacillus*. This pattern reflects the selective pressure of low sulfite levels and the presence of lysozyme. Treatments with reduced sulfite doses showed a clear increase in the relative abundance of LAB, compared with TW, particularly *Oenococcus oeni* in RS1 and a greater proportion of *Apilactobacillus* spp. and *Fructobacillus* spp. in RS2. According to Prusova et al. (2024) [54], alternative treatments with lower sulfite effectiveness showed greater LAB-related metabolic activity, suggesting reduced inhibition. This pattern indicates that lowering sulfite levels favors the growth and persistence of indigenous LAB throughout winemaking. Therefore, the reduced sulfite doses tested in this study enabled the establishment of LAB populations capable of conducting malolactic fermentation.

The addition of low doses of lysozyme with reduced sulfite (RS+L) resulted in a different outcome. In these wines, the relative abundance of *Oenococcus oeni* was noticeably lower than in the RS treatments, whereas *Apilactobacillus* spp. and *Fructobacillus* spp. became the dominant LAB genera. It should be noted that the LAB population in RS+L also decreased in terms of microbial counts. Lysozyme targets the peptidoglycan layer of Gram-positive bacteria, thereby limiting the growth of LAB [55]. The RS+L treatments, therefore, moderate the growth of LAB populations despite reduced sulfite availability. This suggests that low doses of lysozyme can partially compensate for reduced antimicrobial pressure when sulfite levels are lowered, thereby helping to prevent excessive or premature LAB proliferation. As reported by Delfini et al. (2004) [56], based on their cell wall composition, LAB show different degrees of sensitivity or resistance to lysozyme: *Oenococcus oeni* is more sensitive than *Lactobacillus* spp. and *Pediococcus* spp., which are resistant even at high lysozyme concentrations.

The predominance of *Oenococcus oeni* in RS wines confirms its role in malolactic fermentation and wine stabilization [57]. The detection of *Apilactobacillus* spp. in RS+L wines where *Oenococcus oeni* was not dominant is also significant. Members of this genus are facultatively heterofermentative and capable of malolactic fermentation [58]. Their presence

suggests potential functional redundancy within LAB communities, which may ensure fermentation conclusion under stress conditions or alternative winemaking strategies. The occurrence of other LAB genera, especially in wines in which *Oenococcus oeni* is not predominant, suggests the efficient adaptation of these genera to ethanol and low-pH conditions [59].

In contrast, the RS+C treatment showed a distinct bacterial profile, with notably higher richness and the presence of diverse genera, including *Cutibacterium*, *Corynebacterium*, *Pseudomonas*, and *Sphingomonas*. Chitosan exhibits broad-spectrum antimicrobial activity, particularly against Gram-negative bacteria, but its efficacy depends on pH and molecular structure [55]. The detected heterogeneous microbiota may indicate partial inhibition of dominant taxa, thereby allowing minor groups to proliferate. Similar results have been reported in studies evaluating chitosan as a microbial control agent in wine and other fermented beverages [46,60].

Results highlight that sulfur dioxide reduction and the use of antimicrobial substitutes significantly modify the bacterial composition of wines. Reduced sulfite alone promotes LAB dominance, which may facilitate earlier or spontaneous malolactic fermentation. By contrast, the addition of lysozyme in RS+L serves as a selective control strategy, maintaining LAB at moderate levels and potentially contributing to a more predictable fermentation progression and microbial stability. However, the higher diversity observed in chitosan wines warrants further investigation to determine its impact on wine stability and sensory characteristics.

Significant differences in ethanol content were observed, with wines from the C treatment exhibiting the highest ethanol relative to RS, RS+L, and RS+C. Similarly, wines from the C treatment showed the highest pH values and the lowest titratable acidity. Variations in malic and lactic acid concentrations were also recorded depending on the treatment applied. The lower pH observed in RS and RS+C wines could be attributed to the higher activity of LAB, which produce lactic acid that lowers pH, resulting from reduced SO<sub>2</sub> doses [61]. Additionally, the more oxidizing redox conditions associated with lower SO<sub>2</sub> levels could have altered the ionic and acid-base balance of the medium, promoting a slight acidification of the wine [62]. The increase in lactic acid observed in the RS+C and RS+L treatments suggests partial malolactic fermentation occurred during storage, as indicated by the higher LAB count. In contrast, TW wines showed higher malic acid and lower lactic acid, indicating less advanced malolactic fermentation due to the higher total SO<sub>2</sub> dose used. The higher residual glucose content in RS+C wines could indicate lower fermentation efficiency; however, given that the recorded values are low, it can be concluded that this has no practical effect on wine composition [8].

The treatments significantly affected the concentration and composition of phenolic compounds, including total phenols, anthocyanins, and tannins. Traditional winemaking (TW) wines exhibited the highest concentrations of these compounds, whereas the RS+C treatment showed the lowest. These differences are mainly attributable to the antioxidant and protective role of SO<sub>2</sub>, which limits phenolic oxidation by scavenging oxygen and reducing quinones to their native phenolic forms, thereby preventing irreversible degradation and promoting controlled polymerization that contributes to color stabilization [62]. Additionally, sulfur dioxide may enhance the extraction of phenolic compounds from grape skins and seeds by facilitating cell wall disruption and increasing their solubility during maceration [5]. Conversely, reducing SO<sub>2</sub> levels increases the exposure of phenolic compounds to oxidative and condensation reactions, favoring their transformation, polymerization, or removal from the soluble fraction [63]. The higher susceptibility of low-SO<sub>2</sub> wines to color loss is therefore associated with enhanced phenolic oxidation. In the absence of sufficient molecular SO<sub>2</sub>, quinones derived from catechol-type phenols are not efficiently

reduced, allowing metal ion-catalyzed redox cycling ( $\text{Fe}^{3+}/\text{Cu}^{2+}$ ) and subsequent polymerization reactions that decrease anthocyanin stability and promote browning. In addition, the greater microbial activity typically observed under low- $\text{SO}_2$  conditions may intensify these effects through oxidative enzymes such as laccases and peroxidases, which further modify the extraction, stability, and evolution of phenolic compounds during winemaking and ageing [44,64].

The interaction between chitosan and phenolic substances can further explain the particularly low phenolic and anthocyanin contents observed in the RS+C treatment. At wine pH, chitosan carries positively charged amino groups that enable electrostatic adsorption of negatively charged phenolate species and hydrogen bonding with hydroxyl groups of anthocyanins and tannins. These interactions promote aggregation, flocculation, and subsequent precipitation of phenolic complexes, thereby reducing the measurable phenolic fraction in solution and increasing lightness ( $L^*$ ) values [65,66]. Moreover, the higher microbial diversity detected in RS+C wines may have facilitated enzymatic or non-enzymatic oxidative reactions that further intensified phenolic losses. Further fractionation of monomeric and polymeric phenols before and after chitosan treatment would help clarify the relative contribution of adsorption and precipitation mechanisms.

In contrast, RS+L wines showed intermediate values of total phenols, anthocyanins, and tannins, suggesting a moderate effect of lysozyme on phenolic stability. Lysozyme selectively inhibits lactic acid bacteria (LAB) through the hydrolysis of  $\beta$ -(1 $\rightarrow$ 4) glycosidic bonds between N-acetylmuramic acid and N-acetylglucosamine in the peptidoglycan layer of Gram-positive cell walls, leading to cell lysis. This mechanism reduces LAB populations while exerting limited effects on yeasts and Gram-negative bacteria, thereby indirectly limiting the release of  $\beta$ -glucosidase and other degradative enzymes involved in anthocyanin breakdown [67]. However, lysozyme may also interact directly with polyphenols, forming protein-phenol complexes that precipitate or decrease the quantifiable free fraction [68]. Overall, these combined chemical and microbiological effects explain the intermediate preservation of phenolic compounds observed in this treatment.

Regarding the anthocyanin profile, qualitative differences among treatments indicate that both additives and  $\text{SO}_2$  availability selectively modulated pigment stability. Although the RS+C treatment showed the lowest total phenolic concentration, it presented the highest relative proportion of malvidin, whereas RS wines displayed a greater percentage of coumaroylated anthocyanins. These trends suggest selective removal or transformation of specific phenolic fractions. In chitosan-treated wines, differential adsorption of anthocyanins and tannins through electrostatic and hydrogen-bond interactions may reduce the free pigment fraction and alter their relative distribution [65,66]. In contrast, reduced  $\text{SO}_2$  protection enhances the oxidative degradation of less-stable anthocyanins or their conversion into less intensely colored polymeric pigments, thereby altering anthocyanin composition [63,69].

The differences in the chromatic parameters analyzed are attributable to changes in anthocyanin and tannin concentrations in the wines. RS and C wines had a lower  $L^*$ , while RS wines had a higher  $a^*$  value, although not significantly different from TW wines. This may be due to a higher proportion of anthocyanins or the preservation of colored species in these treatments. In contrast, RS+C wines, which presented greater anthocyanin loss, showed higher lightness and lower chroma. The antioxidant function of  $\text{SO}_2$  includes reducing quinones and preventing reactions that lead to the formation of less-colored pigment [70]. The reduction of  $\text{SO}_2$ , together with the possibility of physicochemical interactions with chitosan, would explain the observed color variations. Similar results were observed for the RS+L wines, which exhibited the lowest color in the red-green component.

Sensory analysis revealed significant differences in only two parameters: bitterness and persistence. The absence of significant differences in preference suggests that, despite the physicochemical and microbial alterations detected, the panelists' overall perceptions did not indicate a preference for any treatment. The greater persistence and slightly higher TW scores for certain attributes could be associated with a higher tannin and total phenolic compound content. These compounds are associated with wine structure. The greater bitterness observed in TW and C wines may be attributable to changes in tannin origin or to microbial degradation products. In practical terms, this suggests that alternative strategies, such as RS+L or RS+C wines, may produce detectable sensory changes in specific attributes without necessarily reducing acceptability among trained tasters.

## 5. Conclusions

The results of this study show that the tested strategies for reducing or replacing SO<sub>2</sub> significantly affect the microbiological, physicochemical, and phenolic composition of Tannat wines.

In microbiology, alcoholic fermentation occurred as expected across all treatments, indicating that the evaluated strategies (chitosan, lysozyme, and reduced doses of SO<sub>2</sub>) did not affect the fermentative activity of *Saccharomyces cerevisiae*. However, the post-fermentation microbial dynamics were different. The treatments that included sulfur dioxide showed a marked reduction in lactic acid bacteria populations after bottling. In contrast, those incorporating chitosan or lysozyme maintained, at the same stage, levels of these microorganisms comparable to those in traditional winemaking. Metagenomic analysis revealed lower bacterial diversity in wines treated with sulfur dioxide, whereas wines containing chitosan presented a more complex microbiota. Treatments including lysozyme caused a selective reduction in lactic acid bacteria, primarily affecting *Oenococcus* spp. Partial malolactic fermentation was observed with reduced sulfur dioxide doses when chitosan or lysozyme was present. These results highlight the impact of antimicrobial strategies on bacterial activity and wine acidic balance, potentially influencing its sensory profile and biological stability.

Reducing sulfur dioxide increased the susceptibility of phenolic compounds to oxidation and condensation, whereas adding chitosan could promote interactions that reduced the concentrations of anthocyanins and tannins. Low doses of lysozyme could promote the formation of protein-phenol complexes that may have decreased their concentration. These changes were observed in the colorimetric parameters.

The results suggest that strategies aimed at reducing or replacing sulfites in wines affect not only the microbiota and its dynamics during winemaking but also the acidity, phenolic structure, and color. While the evaluated strategies proved technologically viable, careful process management is required, particularly with respect to microbiological control and phenolic stability, to preserve the wine's sensory quality and varietal identity.

**Author Contributions:** All authors contributed to the development of this manuscript. D.P. and M.G. participated in the study's conceptualization. D.P. and M.G. contributed to the methodological design. The experimental work and laboratory investigations were carried out by D.P., M.G., Y.C.-A., F.P.-F., A.C., T.F.-C., G.F., and J.O. Microbiological analyses and sequencing were performed by T.F.-C., H.N., M.G., and J.O. Data curation was conducted by D.P., M.G., Y.C.-A., F.P.-F., and A.C. Formal analysis was conducted by D.P., M.G., Y.C.-A., F.P.-F., and A.C., D.P. and M.G. wrote the original draft. All authors (D.P., M.G., Y.C.-A., F.P.-F., A.C., G.F., J.O., T.F.-C., H.N., and G.G.-N.) contributed to the review and editing of the manuscript. D.P. supervised the study and was responsible for funding acquisition. All authors have read and agreed to the published version of the manuscript.

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**Institutional Review Board Statement:** In Uruguay, no regulation restricts or prohibits sensory analyses conducted within the framework of scientific research. Nevertheless, the research project was evaluated by experts to ensure compliance with national and international ethical standards. The wines produced were suitable for human consumption in accordance with the regulations established by the National Institute of Vitiviniculture (INAVI). The additives evaluated comply with the International Oenological Codex and were applied at doses lower than those recommended by the OIV for enological use. The sensory evaluation posed no risk because participants did not ingest the wine after tasting; therefore, the study was deemed exempt from formal ethical approval.

**Informed Consent Statement:** All participants were over 26 years of age, participated voluntarily, and were informed about the study's objectives, procedures, the voluntary nature of participation, and the confidentiality measures in place. Verbal informed consent was obtained from participants because the sensory analysis was noninvasive and minimally invasive.

**Data Availability Statement:** The raw sequencing data for this study have been deposited in the Sequence Read Archive (SRA) under the accession number PRJNA1367647.

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## Abbreviations

The following abbreviations are used in this manuscript:

|                 |                        |
|-----------------|------------------------|
| SO <sub>2</sub> | Sulfur dioxide         |
| C               | Chitosan               |
| L               | Lysozyme               |
| RS              | Reduced Sulfites       |
| TW              | Traditional Winemaking |

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