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Nitrogen Sources Added to Must: Effect on the Fermentations and on the Tempranillo Red Wine Quality

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Abstract: Nitrogen supplementation in musts or during the alcoholic fermentation is a common practice to promote fermentations. In this study, the impact of the supplementation of two different sources of nitrogen during Tempranillo red wine elaboration was studied. Mineral and organic nitrogen was added after the exponential yeast growth phase and during winemaking, examining its impact on the alcoholic and malolactic fermentation development, on the aromatic wine composition and on the nitrogenous wine composition. The nitrogen supplementation did not provide neither significant advantages in kinetics and fermentations time, nor differences in the chemical wine composition. The aromatic composition of the wines improved with the addition of inorganic nitrogen, although its organoleptic evaluation was not favored. Moreover, the concentration of amino acids in wines increased slightly after the malolactic fermentation and significantly during the stabilization time, especially with organic nitrogen addition. However, the synthesis of biogenic amines did not increase in wines neither after the malolactic fermentation, nor after the storage period.

Keywords: nitrogen; Tempranillo red wine; alcoholic and malolactic fermentations; amino acids; volatile compounds; wine quality

1. Introduction

Winemaking is a process that frequently involves two successive microbiological stages: The alcoholic fermentation (AF) and the malolactic fermentation (MLF). The first one is conducted by yeasts, typically *Saccharomyces cerevisiae* (*S. cerevisiae*), and the second one is carried out by wine lactic acid bacteria (LAB), mainly *Oenococcus oeni* (*O. oeni*). Both fermentations may occur spontaneously from the activity of yeasts and bacteria naturally present in musts and wines, although currently the winemakers use commercial starter cultures of yeast and LAB for better control of these fermentations.

Nitrogen is the major limiting nutrient for microbial growth under oenological conditions, affecting both fermentation kinetics and the formation of volatile compounds [1]. It is a key nutrient because the yeasts and LAB responsible for wine fermentations have certain basic nitrogen nutrient requirements. Nitrogen in grapes and musts is present as both inorganic (ammonium salt) and organic (protein, peptide, and, mainly, amino acids) forms. Yeast assimilable nitrogen (YAN) is mainly composed of ammonium and amino acids except proline [1]. Yeasts can synthesize all required nitrogen compounds including amino acids, from ammonium, but if amino acids are present in the must, yeasts will use them until ammonium has been depleted. Under enological conditions, yeasts require a least a minimum of 140–150 mg/L of YAN to complete fermentation within a reasonable period of time

and to prevent stuck fermentations [2–5]. But that level is dependent on sugar concentration and winemaking practice [1]. Nevertheless, it strongly depends on the genetic bases of yeast species and clones developing during the fermentation process [6–9]. In this way, some strains consumed nitrogen much more quickly than others [10] so that the selection of yeasts with low nitrogen requirement is a current need in winemaking to avoid stuck fermentations caused by low nitrogen content in grape musts [11]. LAB require only complex organic nitrogen sources, as amino acids. They can also utilize peptides or proteins as nitrogen sources by the breakdown to amino acids by proteolytic enzyme activity. Yeast strains can also secrete certain amino acids into the wine, becoming in nitrogen source for LAB development [12].

Different factors, including grape variety, geographical origin, climate conditions and some technological processes, affect the YAN content in musts and thus the fermentation kinetics [5]. Currently, the most common method for dealing with nitrogen-deficient fermentations is adding supplementary ammonium salts to improve AF. The addition of ammonium in the must imbalances the natural ratio of inorganic/organic nitrogen composition and affects the amino acid uptake pattern [2,10] enhancing the utilization of ammonia and reducing the consumptions of amino acids [13] that would remain residual in the wine before MLF. The residual amino acids in wine favor the growth of wine spoilage microbes, e.g., *Pediococcus* spp. [14] and could act as precursors to the formation of biogenic amines (BAs) by LAB [15], so that an alternative to adding ammoniacal nitrogen is adding organic nitrogen. That is the reason because amino-acid addition has become increasingly common. In addition, it has been demonstrated by some authors that the use of an amino acid mix for must supplementation increases the rate of AF more than ammonium phosphate addition, with sometimes less production of undesirable volatile compounds [16,17].

Aromatic complexity is an essential aspect of wine quality and largely influences consumer's acceptance [18]. Volatile fermentative compounds are synthetized during the AF. The most important compounds include higher alcohols and esters, which are mostly associated with solvent/fusel odors and fruity/floral aromas. Their production is influenced by the yeast strain [19], the fermentation temperature, the available nitrogen for *S. cerevisiae* [20], and the must composition in lipids [21]. Nitrogen supplementation regulates the formation of many volatile and non-volatile compounds that can contribute to the improvement of the final flavor of the wines [22]. Data found in bibliography concerning the impact of YAN addition on volatile compound showed several contradictions related mainly with the source of nitrogen (organic or inorganic) and with the timing of addition. Most of references found about this topic have refer to adding inorganic nitrogen (ammonium salts) at the beginning of fermentation. Nevertheless, [23] indicated that the aroma production was impacted both the timing of the addition and the composition of the nitrogen source.

Most of the studies carried out on nitrogen supplementation in the must have been developed either in the laboratory with synthetic must or with real grape juice in small recipients or in white wine elaborations. The aim of this study was to examine the impact of mineral and organic nitrogen addition on the AF and MLF development and on the Tempranillo red wine quality in semi-industrial elaborations.

2. Material and Methods

2.1. Experimental Design

This study was carried out with grapes of the Tempranillo variety from La Rioja region in Spain (pH 3.21, malic acid 2.54 g/L, 19.34 brix degree, ammonium content 84.5 mg/L and 142.5 mg/L of YAN). The grapes were destemmed, crushed, and homogeneously distributed in six 100-l tanks, sulphited with 50 mg SO_2/kg and inoculated with the yeast Uvaferm VRB®. 72 h after the yeast inoculation, and coinciding with the end of its exponential growth phase, the following treatments were performed in duplicate: addition of inorganic nitrogen (Inorganic N), addition of organic nitrogen (Organic N) and no addition (Control). In the first case, 30 g/hL of $(NH_4)_2HPO_4$ (DAP) were added, which meant a

contribution of 81.8 mg/L ammonia. In the second case, 20 g/hL of a commercial nitrogen activator (Fermaid® O) composed of cell yeast autolysates were added (source of amino acids, vitamins, and minerals). This commercial preparation represented the following increase in amino acids: Aspartic acid (17.4%), glutamic acid (22.9%), glutamine (14.5%), histidine (5.3%), citrulline (8.6%), arginine (15.8%), alanine (11.1%), tyrosine (5.8%), valine (8.5%), and tryptophan (3.4%), while the concentrations of asparagine, glycine + threonine, phenylalanine, isoleucine, and leucine were not modified.

The AF development, at winery temperature, was daily controlled by density decrease. Once the AF was completed, after pumping and pressing, each wine was analyzed, transferred to a 50 L tank, and the Uvaferm β LAB was inoculated. Once the MLF was completed, the wines were also analyzed, transferred to 25 L tanks and sulphited at a dose of 30 mg/L SO_2. After five months of stabilization, the wines were again analyzed and the organoleptic analysis was carried out.

2.2. General Analytical Parameters

Must and wine parameters such as alcoholic strength, pH, sugar, total acidity, malic acid, lactic acid, volatile acidity, color intensity (CI), and hue were determined according to official ECC methods (ECC, 1990) and tartaric acid by the Rebelein method [24]. Citric acid and glycerol were enzymatically analyzed using commercial kits (Boehringer-Manhein). Total phenolics were determined as total polyphenol index (TPI) by spectrophotometric absorbance at 280 nm and total anthocyanins were measured by bleaching using sulfur dioxide [25].

2.3. Aromatic Compound Analysis

Volatile compounds were determined following a method described by [26] with some modifications. The extraction was carried out by mixing 4 mL of sample, 9 mL of $(NH_4)_2SO_4$ saturated solution, 40 µL of internal standard solution (2-butanol, 4-methyl-2-pentanol, 4-hidroxy-4-methyl-2-pentanone, 2-octanol, and heptanoic acid, 40 mg of each/100 mL of ethanol) and 300 µL of dichlorometane in 20 mL tubes. The tubes were shaken for 1 h at 400 rpm. Continuous liquid–liquid extraction was carried out for 1 h, the injection was in split mode and the chromatographic conditions were as follows: Injector temperature: 220 °C; Detector temperature: 280 °C, Oven temperature: 40 °C (5 min), increase of 3 °C/min up to 220 °C, temperature that was maintained for 20 min. The identification of the compounds in the wine was carried out by comparison with the retention times of the standard substances. Quantification was performed by comparing the relative areas of each compound with respect to the area of the internal standard.

2.4. Analysis of Nitrogenous Compounds

Urea and ammonia were determined by enzymatic analysis, using commercial kits (Boehringer-Manhein). YAN was determined by a method based on the combination of the amino function of amino acids with formic aldehyde and subsequent dosing of the free carboxyl by acidimetry [27]. Amino acids and BAs were determined by reverse phase HPLC using a Hewlett Packard Series 1100 liquid chromatograph equipped with an automatic injector (ALS Hewlett Packard Series 1100), a fluorometric detector (Agilent 1100) and a UV-DAD detector (Hewlett Packard UV-DAD Series 1100), applying the methods described by López et al. [28].

2.5. Microorganism Count and Identification

Samples of the fermenting must were plated in chloramphenicol glucose agar medium, using the successive decimal dilutions technique. The plates were incubated at 25 °C for 48 h and counting was done on the plates containing between 30 and 300 colonies. Ten colonies were isolated from plates and identifications at the genus and species level were carried out by PCR-RFLP of the ITS region of the ribosomal DNA. The protocol employed for typing *S. cerevisiae* strains was based in mitochondrial DNA restriction analysis as described Garijo et al. [29].

LAB isolation was performed using the successive decimal dilutions technique and the modified MRS agar culture medium (MRS plus 100 mL/L tomato juice, 6 g/L fructose, 5 g/L D,L malic acid,

0.5 g/L HCl cysteine and 50 mg/L pimaricin) was used. Plates were incubated at 30 °C in anaerobic jars (Gas Pak System) for 10 days, and counting was done on plates containing between 30 and 300 colonies. Ten colonies were isolated from plates and identifications at the genus and species level were carried out by *O. oeni*-specific PCR analysis [30]. Typing of the *O. oeni* clones was performed by Pulsed Fields Gel Electrophoresis technique (PFGE), using the restriction enzyme Sfi *I* and the CHEF-DR III system from Bio-Rad, according to the method described by López et al. [31].

2.6. Sensory Analysis

Eight experienced wine sensory panelists conducted the sensory analysis of wines after 5 months of storage, from the mixture of the two repetitions of each treatment. Wines were randomly presented, served at 18 °C in approved wine glasses (UNE 87-022-92 NORM, AENOR 1997), and were evaluated in individual booths (UNE NORM 87-004-79). Panelists were not informed about the nature of the samples to be evaluated. The analysis was composed of six descriptors groups by visual phase, olfactory phase (flavor intensity and quality), taste phase (taste intensity and quality), and harmony. Panelists rated each attribute on a reverse scale that gives more score to the lower quality [32].

2.7. Statistical Analysis

The statistical study was performed using the analysis of variance (ANOVA). When significant differences were detected ($p \leq 0.05$), the Tukey test was used for the separation of means. The SYSTAT 7.0 statistical program was used.

3. Results and Discussion

3.1. Influence of Nitrogen Addition on the Development of AF and MLF

The addition of nitrogen did not influence neither the development of AF, which ended in all cases in 12 days (Figure 1A) nor the growth of yeasts (Figure 1B). Moreover, the *S. cerevisiae* strains typed by mitochondrial restriction analysis showed that every strain was indistinguishable to the commercial inocula employed, so that establishment of commercial S. cerevisiae strain was the 100% (profiles not shown). These results do not coincide with other studies carried out about this topic, which have reported that nitrogen addition affect fermentation rate either enhancing the fermentation activity or the number of cells [1,10,20]. Varela et al. [33] concluded that the increase in viable cell concentrations positively correlated with the increase in fermentation rates and Seguinot et al. [23] showed that the nitrogen additions systematically reduced the fermentation duration. However, in another work carried out by López et al. [34], in which the addition of different doses (15, 30, and 60 g/hL) of DAP was studied for three consecutive vintages in musts of the Viura variety, the additions did not produce significant modifications neither in the spontaneous fermentative kinetics nor in the number of yeasts. There are several factors such as the YAN present in the must initial, the strain of *S. cerevisiae* used, the sugar concentration and type of nitrogen source, that may have influence in the fermentation profile. This has led to several contradictions depending on the experiments. Moreover, in this work differences were not detected in AF development and in the maximum number of yeasts according to the type of nitrogen added, what agrees with Gobert et al. [5], who found difficult to conclude whether the addition of an amino acid mixture to the must has a better potential to increase biomass formation than ammonium sulfate.

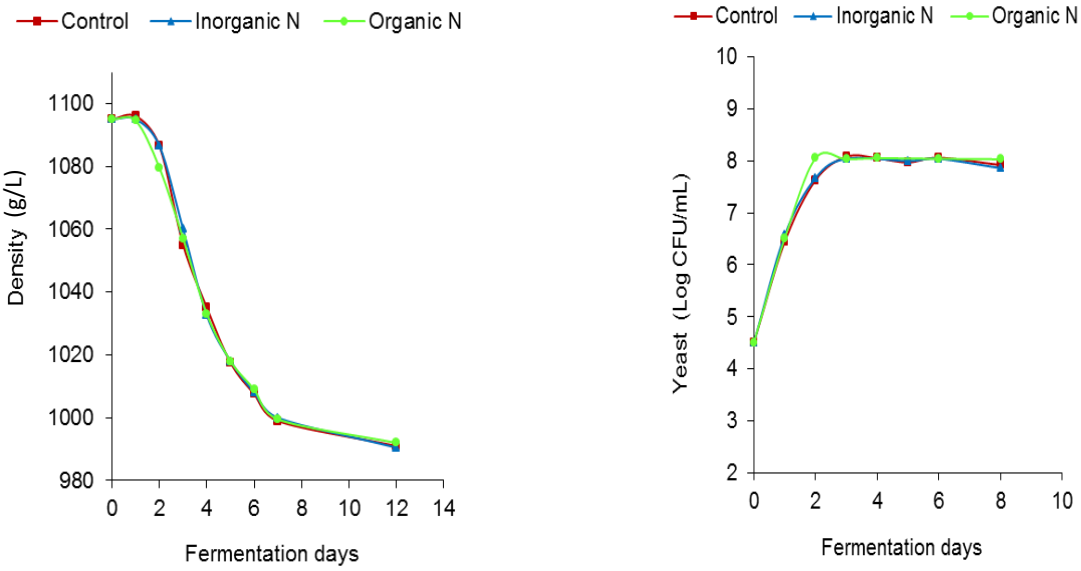

Figure 1. Evolution of density (**A**) and yeast population (**B**) during AF carried out with different nitrogen addition.

The low impact of the addition of nitrogen on the kinetics of AF in this assay could be due to the enough concentration of YAN in the initial must (142.5 mg/L) according to Agenbach [35]. In contrast, this initial amount of nitrogen was low if we consider the levels of 250 mg/L to ferment a must of 23.4° Brix proposed by Bisson et al. [36]. However, it should be noted that the aforementioned limits were established for the production of white wines [37]. In the production of red wines, grape skin maceration can lead to an increase of nitrogen, which makes the amount of YAN available to the yeasts greater than that initially quantified in the must.

The effect of the yeast species and *S. cerevisiae* strains involved in fermentation is also a variable to consider [5] because each strain shows differences in terms of their YAN consumption capacities [38]. Crépin et al. [39] showed that the kinetics of YAN consumption were strongly strain dependent. They tested fourteen *S. cerevisiae* strains in synthetic medium and obtained 40% of the variability in the maximum rate of nitrogen consumption. Gutiérrez et al. [10] indicated that some strains of *S. cerevisiae* showed higher growth-rate and maximum population size and consumed nitrogen much more quickly than others consume. The high nitrogen assimilation rate seems to be a strategy that allowed yeasts successful competition during the growth in grape musts [40]. The selection of yeasts with low nitrogen requirement is a current need in winemaking to avoid stuck fermentations caused by low nitrogen content in grape musts [11,41]. The commercial yeast VRB employed in this study is characterized by a short lag phase and a steady fermentation rate with an assimilable medium nitrogen requirement and a significant release of polysaccharides [42]. These characteristics can justify the previous results.

The addition of nitrogen did not affect the duration of the MLF, which was 23 days, regardless of the treatment (Figure 2A) and it hardly influenced either the degradation rate of malic acid. Except at the end of fermentation, there were also hardly any differences in the LAB during MLF. At this time, their count was four times higher in wines made with the addition of organic nitrogen (Figure 2B). Every LAB strains isolated in tumultuous MLF belonged to the species *O. oeni*. In the control tanks, 100% of the isolated bacteria showed the profile of the inoculated strain. However, in the tanks with nitrogen addition, together with the seeded strain, another clone was detected in great quantity (33 and 44.5%). Both detected genotype were typed as commercial inocula. Consequently, although nitrogen addition could be the cause of lower implantation rate of the selected commercial LAB inocula, indigenous LAB were not found in any case.

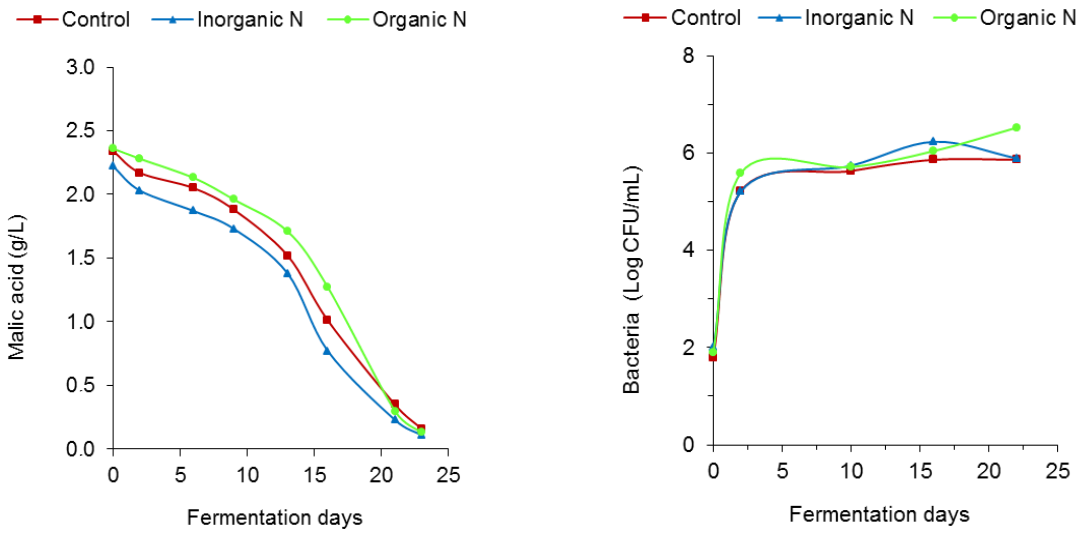

Figure 2. Malic acid degradation (**A**) and lactic acid bacteria (LAB) count (**B**) during the malolactic fermentation (MLF) carried out from wines made with different nitrogen additions.

3.2. Physicochemical Characteristics of Wines

The analysis of the wines carried out after AF indicated that there were hardly any differences between the chemical parameters of control wines and those made with the addition of nitrogen. Only volatile acidity and anthocyanins showed significant differences (Table 1). Although the values of both parameters were significantly higher in wines from organic nitrogen treatment, the absolute differences were small. Regarding volatile acidity, some authors have indicated that ammonium phosphate additions led to the higher production of acetic acid [13,20,22,43] but other authors have reported that adding ammonium sulfate to synthetic must always resulted in lower volatile acidity and [44–46] reported a neutral effect. As regards the concentration of anthocyanins, studies on the impact of nitrogen on the phenolic composition of wine have not been found in the bibliography, perhaps because most of the studies have been done with white or synthetic must. In our work, significant differences were not found neither in pH nor in glycerol content. In this aspect, Perez et al. [20] indicated that the addition of 200 mg/L of ammonium salt showed the lower levels of pH while Chen et al. and Ugliano and Henschke [13,46] reported that ammonium additions led to the higher production of glycerol, but Vilanova et al. and Seguinot et al. showed an insignificant effect [22,23].

About urea concentration, although some authors detected increases in urea in wines when DAP was added [47], in this work significant differences were not observed between the wines. These results can be explained by the low assimilation of arginine, a precursor to urea formation during AF, which would be due to a low concentration of this amino acid in Tempranillo variety, as indicated López et al. [28]. After MLF, every wine had similar characteristics, regardless of treatment (Table 1). The differences in volatile acidity and anthocyanin observed after AF were not maintained at this time. Comparing both moments, it can be seen that during the MFL there was a decrease in all the parameters involved in the color of the wine, as other authors had previously indicated [48].

Table 1. Average values of physicochemical parameters of the wines after alcoholic fermentation (AF) and MLF with different nitrogen additions.

Physicochemical Parameters	After AF			After MLF		
	Control	Inorganic N	Organic N	Control	Inorganic N	Organic N
Ethanol (% *v*/*v*)	13.8	13.7	13.8	13.8	13.7	13.8
Reducing sugars (g/L)	2.15	2.16	2.11	2.15	2.16	2.11
pH	3.53	3.53	3.53	3.59	3.59	3.60
Total acidity (tartaric acid g/L)	7.36	7.25	7.40	6.50	6.03	6.15
Tartaric acid (g/L)	2.73	2.61	2.89	3.11	3.00	3.15
Malic acid (g/L)	2.34	2.22	2.36	nd *	nd	nd
Lactic acid (g/L)	nd	nd	nd	1.48	1.48	1.58
Volatile acidity (acetic acid g/L)	0.16 a	0.15 a	0.20 b	0.21	0.23	0.25
Citric acid (mg/L)	228	231	238	103	92.7	99
Glycerol (g/L)	9.79	11.9	8.50	8.58	8.93	8.35
Color intensity	22.1	22.8	24.0	15.2	15.7	15.7
Hue	0.394	0.393	0.408	0.462	0.466	0.474
TPI	63.2	66.1	66.9	61.6	63.1	62.1
Anthocyanins (mg/L)	1251 a	1232 a	1299 b	1032	1049	1064
Urea (mg/L)	3.76	2.32	3.55	2.73	4.49	4.93

* not detected. Different letters in the same row indicate significant differences according to the Tukey test ($p \leq 0.05$), for the same elaboration stage.

3.3. Aromatic Composition of Wines

Regarding the aromatic composition of the wines after AF, when we added inorganic nitrogen, the wines had a higher content of the 1-propanol, isobutanol, 2 + 3 methyl-1-butanol and 2-phenylethanol alcohols, they had higher concentrations of ethyl-3-hydroxybutyrate and isoamyl acetate esters, and isovaleric acid (Table 2). The increase in the concentrations of the higher alcohols propanol, isobutanol, 2 + 3-methyl-1-butanol, and 2-phenylethanol can be considered favorable for the quality of the wine. 2-Phenylethanol provides a fruity aroma, and the slight increase in higher alcohols provides a desirable level of complexity in wine [49]. The higher content of isoamyl acetate and ethyl-3-hydroxybutyrate esters is also favorable for quality. These compounds, which provide pleasant floral and fruit aromas, derive from the metabolism of sugars and amino acids by yeast. Barbosa et al. [50] also found that isoamyl alcohol production was higher when ammonium was added. However, Martínez et al. [51] found that ammonium salt addition during the stationary growth phase appears to generally decrease ester production and Hernández-Orte et al. [44] did not find significant effect on ester production. With respect to fatty acids, the addition of DAP significantly increased some of them, such as isovaleric acid. These compounds are important in aromatic characteristics, but studies have not been found in the literature on the influence of nitrogenous matter on its concentration.

Nevertheless, significant differences were not found after AF due to the addition of organic nitrogen to the must (Table 2) compared to control. These data differ from those obtained in the same conditions by Seguinot et al. [23] who found that organic nitrogen addition significantly increased the production of propanol and isoamyl acetate, even more than ammonium addition. Differences with our results could be due to the type of nitrogen used in both studies. Most works used amino acids as organic nitrogen, while the source of organic nitrogen employed in our assay was composed of inactive yeast cells and cell autolysates. Fairbairn et al. [16] observed that when the complexity of nitrogen added to the tanks increased, aroma production became unpredictable. Seguinot et al. [23] assessed that propanol production is strongly dependent on the nature of the added nitrogen source (inorganic or organic), and Mouret et al. [52] showed that the addition of ammonium salt induced greater production of propanol than amino-acid addition, in particular, after addition during the stationary phase.

Some authors observed an inverse relationship between the availability of nitrogen and the production of acetaldehyde and diacetyl during AF [53]. However, in this work we have not detected a decrease in the content of these compounds when nitrogen was added to the must (Table 2). The synthesis of carbonyl compounds (diacetyl, 2, 3-butanediol and acetoin) is regulated by the

availability of nitrogen [1]. When the nitrogen content is low, the synthesis of these compounds is activated and it is suppressed when the availability of nitrogen is sufficient. The absence of differences in the concentration of diacetyl and acetoin in the wines again indicated that the availability of nitrogen was sufficient in the initial must.

MLF is an important red winemaking process after AF since it can significantly improve the quality of red wine [48] enhancing the aromatic complexity of wine via LAB metabolism. Our results showed that during the MLF all the wines had a significant decrease ($p \leq 0.05$) of acetaldehyde, and significant increase in octanoic acid, ethyl lactate, methionol, acetoin, diacetyl, and butyrolactone (statistical results not shown), which supposed a greater aromatic complexity of the wines. Bartowsky et al. [54] found the same compounds changing during MLF. The production of diacetyl, esters, alcohols, and other carbonyl compounds results in buttery, fruity, spicy, vanilla, and smoky notes, as well as a softer mouthfeel [55]. In many cases, the specific changes are due to the lactic acid bacteria driving MLF. Differences between different nitrogen addition treatments in the concentration of higher alcohols and esters after AF (Table 2) were maintained after MLF, which means that the source of nitrogen added had not influence in aromatic compounds formed in MLF.

Table 2. Average values of aromatic compounds of the wines after AF and MLF with different nitrogen additions.

Aromatic Compounds (mg/L).	After AF			After MLF		
	Control	Inorganic N	Organic N	Control	Inorganic N	Organic N
Alcohols						
1-Propanol	20.0 a	26.8 b	20.6 a	19.0 a	24.4 b	18.4 a
Isobutanol	34.4 a	42.6 b	34.2 a	32.6 a	38.9 b	32.4 a
1-Hexanol	2.12	2.35	2.17	1.99	1.92	1.99
2 + 3-Methyl-1-butanol	230 a	291 b	237 a	226 a	268 b	228 a
2-Phenyl ethanol	51.2 a	61.2 b	53.6 a	46.1	48.4	48.9
cis-3-hexenol	0.40	0.47	0.37	0.37	0.38	0.34
Methionol	0.15	0.15	0.16	0.26	0.31	0.30
Benzyl alcohol	0.10 b	0.05 a	0.05 a	0.09 b	0.06 a	0.05 a
Esters						
2-Phenylethyl acetate	0.27	0.33	0.2	30.21	0.26	0.21
Diethyl succinate	2.51	2.08	2.39	2.17	2.01	2.60
Ethyl-3 hidroxybutyrate	0.87 a	1.00 b	0.82 a	0.80 a	0.92 b	0.84 a
Ethyl acetate	48.5	50.4	45.5	51.6	48.6	53.1
Ethyl butyrate	0.32	0.37	0.28	0.33	0.39	0.32
Ethyl hexanoate	0.25	0.27	0.15	0.18	0.20	0.20
Ethyl isobutyrate	0.01	0.02	0.01	0.02	0.04	0.03
Ethyl lactacte	1.15	1.20	1.23	25.2	25.5	26.2
Ethyl octanoate	0.07	0.05	0.02	0.06	0.12	0.14
Ethyl propionate	0.10	0.15	0.09	0.12	0.15	0.12
Hexyl acetate	0.06	0.19	0.13	nd	nd	nd
Isoamyl acetate	3.78 a	4.69 b	3.26 a	3.41 a	3.98 b	3.55 a
Acids						
Butyric acid	2.02	2.14	1.89	1.79	1.98	1.90
Isobutiric acid	2.47 a	3.09 b	2.72 ab	2.82 a	3.42 b	2.83 a
Isovaleric acid	1.94 a	2.38 b	1.97 a	1.74 a	2.09 b	1.87 a
Hexanoic acid	3.42	3.42	3.62	3.39	3.12	3.63
Octanoic acid	1.79	1.56	1.76	2.11	2.22	2.21
Decanoic acid	0.18	0.21	0.13	0.21	0.22	0.20
Other compounds						
Acetaldehyde	7.04	7.91	7.34	2.79	1.79	1.61
Acetoin	0.75	1.01	0.90	4.82	4.62	4.38
Diacetyl	1.15	1.31	1.49	3.02	2.64	2.45
Butyrolactone	1.57	1.59	1.40	2.85	3.31	3.18

Different letters in the same row indicate significant differences according to the Tukey test ($p \leq 0.05$), for the same elaboration stage.

3.4. Nitrogen Composition of Wines

3.4.1. Amino Acids

Table 3 shows the average amino acid composition in the must just after destemming and crushing of grapes, in the wines at the end of AF and MLF, and after five months of conservation. The most abundant amino acids in the must were proline, glutamic acid, arginine, and glutamine, followed by alanine, valine, tryptophan, and serine. Cystine, lysine, or ornithine were not detected. Arginine and proline are usually the majority amino acids in the must and the proline/arginine relation is considered very high in Tempranillo variety [28]. The concentration of the precursor amino acids of BAs (histidine, arginine, tyrosine, phenylalanine and lysine) was low.

During AF, most of the amino acids were consumed in high proportion. This consumption led to an average decrease (less proline) of 81%. In all treatments, aspartic acid, serine, glutamine, arginine, valine, methionine, and tryptophan were the most widely used amino acids (more than 90%). Glutamic acid, histidine, alanine, tyrosine, phenylalanine, isoleucine, and leucine, were consumed between 62 and 85%. Glycine + threonine decreased less than 50%, while cysteine and proline increased their concentration.

Despite the addition of nitrogen, significant differences were not observed between the wines in the amino acid concentration at the end of the AF. This implies that there was a higher consumption of nitrogen in the added wines. These results are in agreement with those obtained by Ferreira-Monteiro et al. [56], who observed that the addition of DAP in a rosé must did not affect the amino acid concentration in the wine.

The high consumption during AF caused the amino acid content (less proline) before the MLF was low compared to other results [57]. However, low concentration of amino acids before the MLF did not impede its development. The most abundant amino acid after AF was glutamic acid, an essential amino acid for LAB, since it is required for the synthesis of other amino acids, purines and pyrimidines [58]. Glutamic acid was the only amino acid that was consumed significantly during MLF (Table 3). However, the tyrosine, phenylalanine, and leucine content increased in all wines. When organic nitrogen was added, glycine + threonine and alanine also increased. Lorenzo et al. [59] also showed how amino acids supplementation of Monastrell must was able to influence the content of amino acids in wine. However, during MLF, each amino acid evolved in a different way, not showing a common trend. There is a disparity of results in relation to the effect of MLF on amino acid concentration in wines. In some cases was observed an increase [60] while in others a decrease was detected [61]. After the MLF, the wines with organic nitrogen added had significant higher concentrations of glutamic acid, histidine, threonine, alanine, phenylalanine + isoleucine, and leucine (statistical results not shown), which could explain the higher LAB population in those wines (Figure 2B).

After the stabilization time, most of the amino acids had increased (Table 3), except glutamine, citrulline, tryptophan, and proline that remained constant and cysteine that decreased. In wines made with the addition of organic nitrogen, the increase was greater and, consequently, after five months, these wines presented higher concentrations of most of the amino acids (statistical results are not shown). These increases could be due to the autolysis of the microorganisms that had participated in the previous fermentations.

Table 3. Average values of the amino acids content during the production and preservation of wines made from musts with different nitrogen additions.

Amino Acids(mg/L)	Elaboration Stage				
	Treatment	**Must**	**After FA**	**After MLF**	**After 5 Months**
Aspartic acid	Control	9.62 c	nd * a	nd a	2.42 b
	Inorganic N	9.62 c	nd a	nd a	2.40 b
	Organic N	9.62 c	nd a	nd a	3.19 b

Table 3. *Cont.*

Amino Acids(mg/L)	Elaboration Stage				
	Treatment	**Must**	**After FA**	**After MLF**	**After 5 Months**
Glutamic acid	Control	31.4 c	11.4 b	6.28 a	9.22 b
	Inorganic N	31.4 c	9.65 b	5.77 a	7.98 b
	Organic N	31.4 c	10.1 b	8.01 a	11.5 b
Asparagine	Control	1.98 a	2.61 ab	2.77 ab	3.37 b
	Inorganic N	1.98	2.94	3.02	3.76
	Organic N	1.98 a	2.80 ab	3.14 bc	4.18 c
Serine	Control	11.4 c	0.76 a	1.00 a	1.67 b
	Inorganic N	11.4 b	1.05 a	1.33 a	1.71 a
	Organic N	11.4 c	1.03 a	1.37 ab	2.09 b
Glutamine	Control	25.3 b	2.56 a	2.47 a	1.18 a
	Inorganic N	25.3 b	2.60 a	1.99 a	1.12 a
	Organic N	25.3 b	2.52 a	1.98 a	1.18 a
Histidine	Control	5.41 b	1.30 a	0.86 a	2.42 a
	Inorganic N	5.41 b	1.30 a	1.41 a	2.53 a
	Organic N	5.41 c	1.21 a	1.75 ab	3.14 b
Glycine + Threonine	Control	8.04 b	4.17 a	4.79 ab	7.61 ab
	Inorganic N	8.04 b	4.00 a	5.59 ab	8.52 b
	Organic N	8.04 c	3.82 a	6.65 b	9.91 c
Citruline	Control	1.76	1.72	1.44	1.15
	Inorganic N	1.76	0.97	1.15	1.15
	Organic N	1.76	0.96	1.47	1.19
Arginine	Control	30.5 b	1.18 a	1.18 a	2.06 a
	Inorganic N	30.5 b	1.51 a	1.01 a	2.22 a
	Organic N	30.5 b	1.07 a	1.95 a	3.60 a
Alanine	Control	13.7 c	4.18 a	5.43 ab	7.26 b
	Inorganic N	13.7 c	4.53 a	5.90 ab	7.84 b
	Organic N	13.7 d	4.48 a	7.07 b	9.76 c
Tirosine	Control	1.80 b	0.56 a	1.37 b	1.78 b
	Inorganic N	1.80 b	0.83 a	1.44 b	1.95 b
	Organic N	1.80 b	0.65 a	1.63 b	2.39 b
Cystine	Control	nd a	1.48 b	1.47 b	nd a
	Inorganic N	nd a	1.32 b	1.51 b	nd a
	Organic N	nd a	0.91 b	1.55 b	nd a
Valine	Control	12.1 c	nd a	nd a	1.63 b
	Inorganic N	12.1 c	nd a	nd a	1.71 b
	Organic N	12.1 c	nd a	nd a	2.18 b
Methionine	Control	1.79 c	nd a	nd a	0.93 b
	Inorganic N	1.79 c	nd a	nd a	0.98 b
	Organic N	1.79 c	nd a	nd a	1.24 b
Tryptophan	Control	11.4 b	0.99 a	1.43 a	1.10 a
	Inorganic N	11.4 b	1.29 a	1.17 a	1.11 a
	Organic N	11.4 b	0.74 a	1.20 a	1.32 a
Phenylalanine	Control	3.36 c	0.54 a	1.77 b	3.13 c
	Inorganic N	3.36 c	0.74 a	1.84 b	3.44 c
	Organic N	3.36 c	0.25 a	2.32 b	4.08 d
Isoleucine	Control	2.77 d	0.27 a	0.89 b	1.27 c
	Inorganic N	2.77 c	0.78 a	1.02 a	1.33 a
	Organic N	2.77 c	0.18 a	1.21 b	1.67 b

Table 3. *Cont.*

Amino Acids(mg/L)	Elaboration Stage				
	Treatment	Must	After FA	After MLF	After 5 Months
Leucine	Control	2.88 bc	0.56 a	2.70 b	3.48 c
	Inorganic N	2.88 bc	0.78 a	2.44 b	3.80 c
	Organic N	2.88 b	0.52 a	3.41 b	4.87 d
Lysine	Control	nd a	nd a	nd a	6.36 b
	Inorganic N	nd a	nd a	nd a	8.74 b
	Organic N	nd a	nd a	nd a	7.77 b
Sum of amino acids-proline	Control	175.21 b	34.28 a	35.85 a	58.04 a
	Inorganic N	175.21 c	34.29 a	36.59 a	62.29 b
	Organic N	175.21 d	31.24 a	44.71 b	75.26 c
Proline	Control	338 a	564 b	613 b	624 b
	Inorganic N	338	456	482	490
	Organic N	338 a	564 b	605 b	639 b

* not detected, lower than the detection limit. Different letters in the same row indicate significant differences according to the Tukey test ($p \leq 0.05$).

3.4.2. Biogenic Amines

Currently, MLF is considered as one of the most important factors that determine the presence of BAs in wines [62]. High concentrations (1–100 mg/L) of BAs can cause undesirable physiological effects in sensitive humans, especially when alcohol and acetaldehyde are present [55]. Putrescine, histamine, tyramine, and cadaverine have been identified as the most abundant BAs found in wine [63]. However, in our work, the content of BAs in wines after MLF was very low (Table 4) in relation to data obtained in other studies on red wines [64]. Only detectable amounts of ethylamine, putrescine and cadaverine were found, with putrescine being the most abundant. At this point, significant differences were not found in the content of the amines depending on the addition or not of nitrogen to the musts (Table 4), what agrees with Smith et al. [65]. However, Bordiga et al. found that BAs appeared significantly related to the nutrient supplementation applied [66] and Lorenzo et al. [59] showed how amino acids supplementation of Monastrell must were able to influence the content of tyramine, histamine, 2-phenylethylamine, and tryptamine.

Table 4. Average values of biogenic amines content of wines after MLF and after 5 months of conservation with different nitrogen additions.

Biogenic Amines(mg/L)	After MLF			Five Months after Final MLF		
	Control	Inorganic N	Organic N	Control	Inorganic N	Organic N
Histamine	nd *	nd	nd	nd	nd	nd
Ethylamine	1.05	1.16	0.99	1.02	1.10	1.08
Tyramine	nd	nd	nd	nd	nd	nd
Phenylethylamine	nd	nd	nd	nd	nd	nd
Putrescine	2.05	1.88	2.36	2.12	1.96	2.16
Isoamylamine	nd	nd	nd	nd	nd	nd
Cadaverine	0.33	0.33	0.33	0.34	0.35	0.34
Sum of biogenic amines	3.43	3.37	3.68	3.48	3.41	3.58

* not detected (lower than detection limit).

The low production of BAs at the end of the MLF could be related to the low concentration of amino acids after AF. Moreno-Arribas et al. [15] showed that high concentrations of amino acids after AF can cause a significant amount of BAs after MLF. Contrarily, Martínez-Pinilla et al. [67] did not find correlation between total amino acids in the medium and total BAs after MLF. Another reason to explain the low content of BAs, could be in the LABs that carried out the MLF [66] because BA formation is strain-dependent [68]. The strategy mostly used nowadays by winemakers to prevent BA

formation is to inoculate safe malolactic starters [15,63]. Currently, all commercial strains have been selected for their inability to produce BAs, which would explain the low formation of amines in all wines. In this study, as it was mentioned above, all LAB strains isolated in tumultuous MLF belonged to the species *O. oeni*. In tanks without nitrogen addition, 100% of the isolated LAB was identified as the commercial inoculated strain but in wines with nitrogen addition, other genotype was detected in percentages higher than 30% and corresponded to another LAB inocula usually employed in the experimental winery.

Extended lees contact also seems to be responsible for higher BAs concentrations in wines [55]. LABs present in wines can hydrolyze and decarboxylate higher levels of peptides and free amino acids released by yeasts [62]. However, in our data there were no significant modification of any of the amines after the five months of wine storage, and no amines other than those found at the end of the MLF were detected (Table 4). These results can be due to three reasons. Firstly, the absence of LAB in wines at five months (viable LAB were not found at this time in any of the wines). Secondly, the low concentration of amino acids in the wines, even after the increase produced during the conservation period and thirdly, the presence of citric acid in high concentrations after five months (69.5, 65.5, and 81.0 mg/L in the control, inorganic and organic nitrogen addition respectively). These amounts would indicate that LABs did not need to decarboxylate amino acids to obtain energy, since microorganisms use this mechanism when they do not have other sources such as sugars, malic acid and citric acid [69]. Landete et al. [70] observed that the presence of citric acid inhibited the expression of the "hdc" gene.

3.5. Organoleptic Analysis of Wines

The results of the organoleptic evaluation of the wines are shown in Figure 3. As it can be seen, there were not differences in visual quality between the wines from different treatments. Nevertheless the wine made with the addition of organic nitrogen was the worst rated in the rest of attributes. When comparing control wine with that made with inorganic nitrogen addition, not many differences were observed, although except for taste quality, control wine was the best valued. The panelists did not detect in this control wine aromatic deviations produced by sulfur compounds, a situation that usually occurs in nitrogen deficiency states. On the contrary, [71] indicated that addition of nitrogen sources in must led to wines rated higher in intensity for most of the fruity and floral attributes, but the type of nitrogen added had no significant effect on the sensorial profile.

The role of nitrogen during wine fermentation has been extensively studied; particularly the effect of nitrogen on the production of volatile compounds, but few studies evaluated its impact of nitrogen addition on wine sensory profiles wines [5]. Differences in aromatic compounds detected in our wines after MLF do not explain the organoleptic differences obtained, and our results contradict the information recovered in bibliography. In this sense, Ferrari et al. [72] found a correlation between assimilable nitrogen in the must and the quality of the wine, linked to the content of higher alcohols, hexanol, esters, and acetates, as well as to aromatic deviations produced by sulfur compounds in nitrogen deficiency states. In the same way, Ugliano et al. [73] indicated that Shiraz wines elaborated with inorganic nitrogen addition to the must had higher scores for the flavor phase and Hernández-Orte et al. [74] valued better the flavor of the wines of the Airén variety from musts supplemented with inorganic nitrogen.

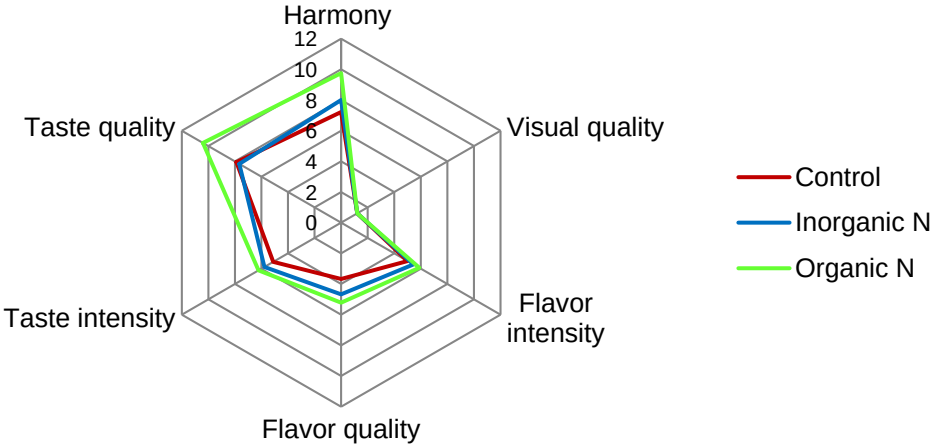

Figure 3. Sensory spider diagram of the different wines elaborated after 5 months of final MLF. Minor score indicated better quality of each attribute.

4. Conclusions

In this study, the addition of nitrogen during the elaboration of red Tempranillo wines, both in inorganic and organic form, did not provide significant advantages in the development of AF or MLF. In the latter, the addition of nitrogen made difficult inoculated bacterial strain establishment. Significant differences were not found between the analytical composition of the control wines and those made with the addition of nitrogen. Despite the fact that the aromatic composition of the wines after AF and MLF improved with the addition of inorganic nitrogen, this result was not reflected in the sensory analysis carried out five months after the end of the MLF.

Differences were not detected in the amino acid concentration in the wines after AF, which implies that there was a higher consumption of nitrogen in the supplemented wines. However, there was a slight increase after MLF and a significant increase during stabilization, especially when organic nitrogen was added. These latter wines had a higher amount of some amino acids, including histidine, which increases the risk of formation of BAs. However, there was no significant modification of any of the amines after a storage period of five months, which could be explained by the absence of LAB, by the low concentration of amino acids and by the presence of citric acid.

It can be concluded that the systematic addition of nitrogen in must to improve the fermentation development and wine quality, is not always justified. It is important to know the nitrogen content of grape juice and the nitrogen requirement for each specific yeast and bacteria strains, in order to achieve optimal fermentations performances. In addition, it must be taken into account that the minimum values necessary for the growth of yeasts proposed in the bibliography are generally extracted from works carried out using synthetic musts or in virgin elaborations. In red winemaking, the contact of the solid parts of the grape harvest with the must during the maceration process can allows the nitrogenous matter present in the skin to be extracted. In many cases, uncontrolled additions may lead to the accumulation of nitrogen in the wines and microbial instability.

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