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ORIGINAL ARTICLE

Effect of nitrogen addition during alcoholic fermentation on the final content of biogenic amines in wine

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Abstract One aspiration of the wine industry is to control technological factors in order to produce wines with low biogenic amines content. Among these factors, amino acids and ammonium ions are essential nutrients for the growth of yeasts and lactic acid bacteria during alcoholic and malolactic fermentation, but they are also potential biogenic amine precursors. Nitrogen is often a limiting nutrient for *Saccharomyces cerevisiae* during batch alcoholic fermentation and must occasionally be modified. This action, however, can be contradictory with the aim of controlling biogenic amine content. Rationalised to nitrogen addition, fermentation experiments at the pilot scale (100 L) were performed using grapes (Syrah and Grenache) obtained from the Rhone Valley, by varying the concentration and type of nitrogen added. The purpose of this work was to assess the effect of nitrogen addition on the final concentration of biogenic amines under wine-making conditions. We showed that, in fact, the addition of nitrogen allows rapid fermentation, limiting bacterial growth. The impact of this supplement, however, is an enrichment of precursors. Our results demonstrate that these two opposing mechanisms are translated into reality by increasing the final concentration of biogenic amines.

Keywords Wine · Fermentation · Nitrogen · Biogenic amines

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Introduction

The occurrence of biogenic amines in fermented foods, such as cheese, sausages, beer and wine, is mainly due to the decarboxylation of certain amino acids by the action of micro-organisms. Histamine, tyramine, putrescine, cadaverine and phenylethylamine are the biogenic amines most frequently found in wines from the respective decarboxylases activities of lactic acid bacteria during malolactic fermentation (Önal 2007). Biogenic amine content in wine is a serious problem from a toxicological point of view, because these amines can cause adverse physiological reactions in susceptible individuals, such as headache, nausea, hypotension reactions or hypertension, heart palpitations, and anaphylactic shock (Bodmer et al. 1999; Jansen et al. 2003). Biogenic amines are of increasing interest to the wine industry due to proposed regulatory issues. Currently, there are ongoing discussions in the European Union regarding regulation of biogenic amines in imported wine. The prevailing opinion is that the presence of biogenic amines in wine indicates poor winemaking practice. A recent EU proposal intends to include biogenic amines under similar regulations proposed for allergens. Switzerland had a published tolerance value for histamine in wine at 10 mg/L, but has recently suspended that and is waiting for the EU to provide a regulatory framework for biogenic amines. Thus, in the future, high levels of biogenic amines could be an obstacle to the marketing of certain wines.

Thus, one of the wishes of the wine industry is to produce wines with low levels of biogenic amines by controlling the critical technical factors. The production of biogenic amines in wine depends not only on the presence of potential biogenic amine-producing bacteria but also on other parameters such as the presence of amino acid precursors, pH, or duration of malolactic fermentation (Martin-Alvarez et al. 2006). Thus, among these factors, amino acids and ammonium ions are

essential growth factors for yeast and lactic acid bacteria during alcoholic fermentation and malolactic fermentation. Nitrogen is often a limiting nutrient in *Saccharomyces cerevisiae* during alcoholic fermentation. Nitrogen, and notably yeast available nitrogen (YAN), is essential for a successful fermentation, insufficient nitrogen levels in musts lead to sluggish or even non-existent alcoholic fermentation. Various studies have shown that a minimum of 120–140 mg YAN/L was required to yield optimum fermentation kinetics (Sablayrolles et al. 1996). It is recommended that these deficient musts should be nitrogen-supplemented to ensure good fermentation (Bely et al. 1990). The most common intervention applied is the addition of ammonium to increase nitrogen content, within the legal limit of 1,000 mg/l in Europe and 950 mg/l in the USA (Taillandier et al. 2007) of diammonium phosphate (DAP) or sulphate. Nitrogen amount is important, but the quality of the nitrogen source is equally important. Each day, research is conducted into new additives and it is therefore of interest to know how the addition of nitrogen sources affects amino acid uptake, particularly of those related to the production of higher alcohols and their corresponding esters, and how this addition affects the synthesis of these aroma compounds (Hernandez-Orte et al. 2006).

These operations, however, may contradict the goal of controlling biogenic amine content, as the addition of nitrogen represents an additional source of potential biogenic amine precursors. To streamline the addition of nitrogen, pilot-scale (100 L) fermentation experiments were conducted using grapes from the Rhone Valley (Syrah and Grenache). Amino acid and biogenic amine levels were monitored, along with bacterial populations. The purpose of this study was to evaluate the effect of nitrogen and amino acids on biogenic amine content in the final wine.

Materials and methods

Materials Pilot-scale (100 L) fermentations were performed using grapes from the Rhône Valley (Syrah and Grenache). The must composition is shown in Table 1. A deficient must was selected (75 mg of available nitrogen/L) and adjusted to 150 mg YAN/L, 300 YAN mg/L and 450 mg YAN/L by using 75 mg N/L, 225 N mg/L and 385 N mg/L of di-ammonium phosphate (DAP), respectively. For each level of nitrogen, an amount of complex nitrogen form (200 mg/L) was added using inactivated dry yeast (commercial product). Finally, an experiment was conducted during which only inactivated dry yeast (400 mg/L) was added. All fermentations were performed in duplicate.

The yeast was inoculated to 200 mg/L with Lalvin Rhône 2056. Similarly, to be sure of the presence of potential biogenic amine-producing bacteria, the bacterial strain *Oenococcus oeni* IOEB 0605 HDC + producing histamine (ISVV, Bordeaux) was inoculated to 10^3 CFU/mL, 24 h after vatting, to minimise the SO_2 effect. The presence or absence of genes required for the production of histamine was monitored during fermentation by quantitative PCR according to the method developed by the University of Bordeaux (Lucas et al. 2008).

Table 1 Syrah and Grenache must chemical analysis

Grape must composition	Syrah	Grenache
Malic acid (g/L)	4.8	3.0
Tartric acid (g/L)	4.7	3.8
Titrable Acidity (H_2SO_4 equivalent g/L)	5.3	4.0
Yeast nitrogen assimilable (mg/L)	75.0	75.0
pH	3.2	3.2
Potassium (mg/L)	1,531.0	1,079.0
Sugar	190.0	236.0

Analysis of amino acids and biogenic amines The protocol used was that developed by Gomez-Alonso et al. (2007) as modified by Inter Rhône.

For the derivatisation reaction, aminoenone derivatives were obtained by the reaction of 1.75 mL of 1 M borate buffer (pH 9), 750 μL of methanol, 1 mL of sample 20 μL of internal standard (L-2-aminoadipic acid, 1 g/L), and 30 μL of diethyl ethoxymethylenemalonate (DEEMM) for 30 min in an ultrasonic bath. The samples were then heated to 70°C for 2 h to allow complete DEEMM breakdown.

For the HPLC analysis, analyses were carried out were on a Varian ProStar HPLC. Chromatographic separation was performed by using an Alltima HPLC column (C18) (5 μm – 250 mm × 4.6 mm) thermostated at 16°C and coupled with a diode array detector.

Culture conditions The lactic acid bacteria populations were monitored on modified MRS medium (Biokar) at 25°C.

Results and discussion

All fermentations were performed without incident, whatever the variety. Despite severe nitrogen starvation, no stopped fermentations were observed, and all sugar was consumed for each treatment used. As expected, fermentation times were reduced in relation to nitrogen intake (Fig. 1). It is worth noting that the shortest fermentation times (10 days) were obtained with a dose of 300 mg YAN/L. Fermentation kinetics remained stable for higher additions. It should be noted that the time reduction obtained with the addition of a

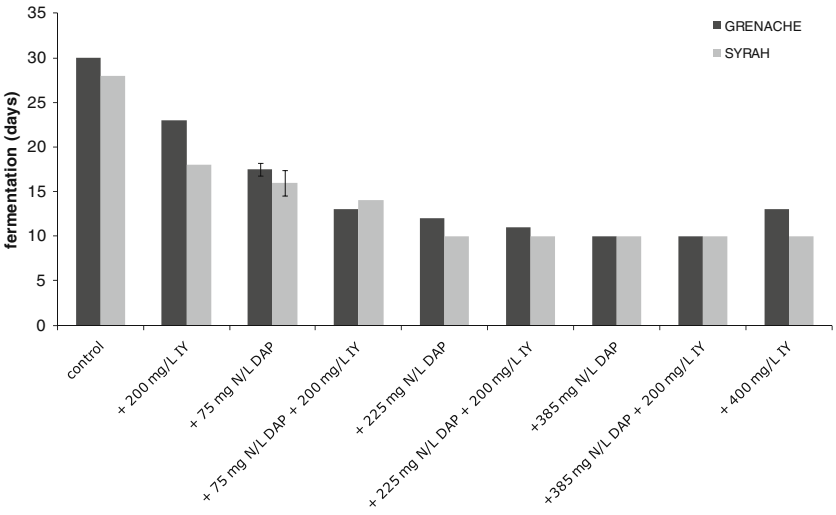

Fig. 1 Duration of alcoholic fermentation observed depending on grape (Syrah □, Grenache ■) and added nitrogen (diammonium phosphate, *DAP*, and/or inactivated yeast, *IY*). Control (natural levels of nitrogen in must)=75 mg N/L. (n=2 independent experiments)

complex form of nitrogen (inactivated dry yeast) was with a dose of 400 mg/L, a quantity usually used in enological practices. This can be explained by the fact that the inactivated dry yeast may include a soluble fraction mostly formed by yeast cytoplasm metabolites (proteins, peptides, amino acids, polysaccharides: glucanes, mannoproteins, sterols and fatty acids), along with an insoluble fraction of inactive inert support, mainly cellulose in their composition. Additionally, other compounds, such as vitamins and minerals, are often included in most inactivated yeast preparations (Pozo-Bayón et al. 2009).

It has been well observed that the bacterial population at the end of alcoholic fermentation is more important when the fermentation was slow to finish due to nitrogen deficiency (Fig. 2). For both cultivars, as nitrogen addition increased, the level of lactic acid bacteria in alcoholic fermentation populations decreased. In all cases, different behaviors were observed according to the variety: the population of lactic acid bacteria in Grenache was higher (about 1 log) than in Syrah. Thus, bacterial populations tended to grow more easily on Grenache musts. Populations of lactic acid bacteria were monitored during fermentation (Fig. 3). During alcoholic fermentation, the bacterial population increased, particularly during the last stage, when yeast activity slowed down, as expected.

At the end of alcoholic fermentation, biogenic amines (histamine, tyramine, putrescine, cadaverine and phenylethylamine) were not detected (<0.5 mg/L) indicating that biogenic amine-producing lactic acid bacteria did not impact biogenic amine levels during alcoholic fermentation, despite their development. Moreover, the inoculated bacterial population did not metabolise malic acid during alcoholic fermentation.

When all sugars were consumed, wines were placed at 10° C to block malolactic fermentation, simulating a "winter effect". This manipulation was done to permit fermentation synchronisation and to allow a better malolactic fermentation monitoring. After this cold period, wines were transferred to 5-L flasks and placed at 18°C in order to achieve the malolactic fermentation. Malolactic fermentation took place over a period of 15–20 days irrespective of the treatment used

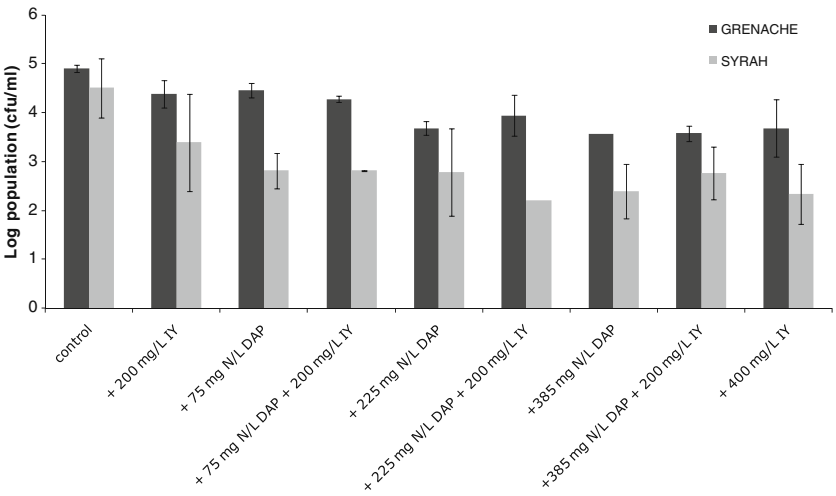

Fig. 2 Bacterial population at end of alcoholic fermentation, depending on grape (Syrah □, Grenache ■) (n=2 independent experiments)

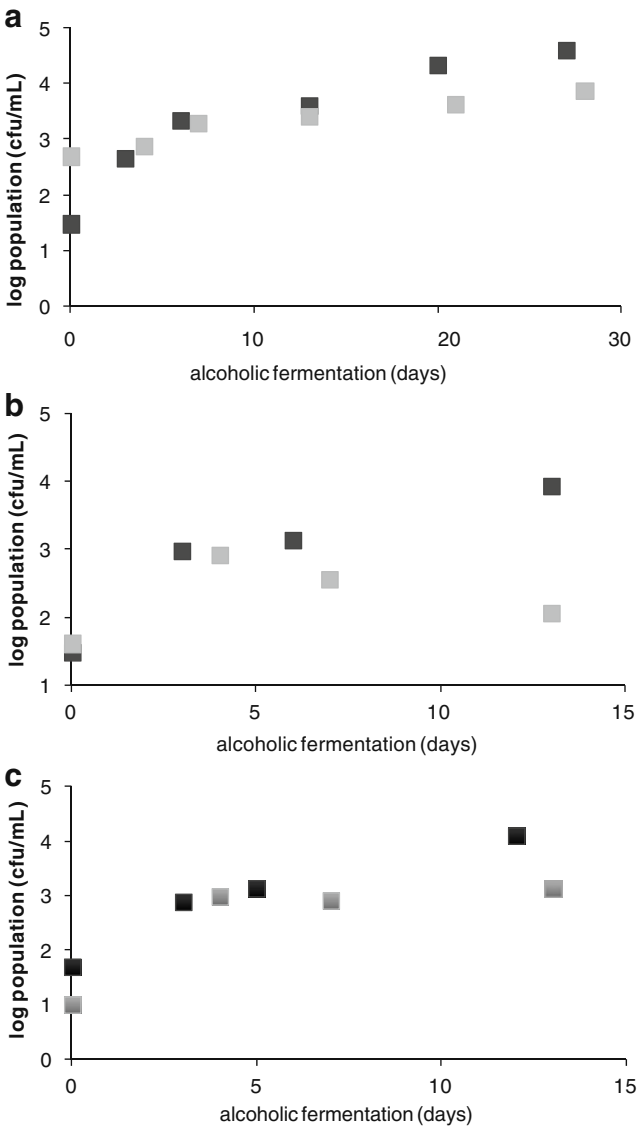

Fig. 3 Population kinetics of lactic acid bacteria during alcoholic fermentation (days) depending on grape (Syrah □, Grenache ■) and added nitrogen (**a** control 75 mg N/L, **b** +225 mg N/L of DAP, **c** +400 mg/L with inactivated yeast)

(data not shown). Malolactic fermentations completed rather quickly and malic acid completely metabolised, suggesting that lactic acid bacteria growth inhibitors were not produced or that the bacteria present were not sensitive. Production of such inhibitors has, however, been previously reported during sluggish fermentation processes (Alexandre et al. 2004). It would seem that lactic acid bacterial growth inhibitors, usually produced by yeasts during sluggish fermentation, were not produced in this case. The characterisation of HDC + lactic acid bacteria was performed by quantitative PCR after completion of malolactic fermentation. At the end of malolactic fermentation, 90–95% of bacteria were potentially producing histamine. The strains involved, however, were not identified; it could therefore have been either the inoculated strain or strains present in the native must.

Biogenic amine content was determined once breakdown of malic acid was complete (Fig. 4). Tyramine, putrescine, cadaverine and phenylethylamine could not be detected (<0.5 mg/L). The histamine content from malolactic fermentation, however, increased dramatically, reaching nearly 10 mg/L under certain conditions. Moreover, significant differences were observed between methods. It has been clearly shown that the addition of nitrogen leads to histamine increase in wine. Similarly, the type of addition seems to be important to fermentation behaviour. For instance, the addition of 400 mg/L of inactivated yeast increased the level of histamine and shortened the fermentation period. It seems that the addition of complex forms of nitrogen, i.e. amino acids, leads to the formation of more precursors.

We have been able to correlate the amount of nitrogen added to the amount of histamine produced. It is interesting to relate these data to the amounts of precursors present at the end of fermentation. In our case, we determined the amounts of histidine, a histamine precursor. No significant correlation, however, could be demonstrated. Histidine levels did not appear to increase with nitrogen addition. It is nevertheless worth noting that the amino acid assay that we used only allows the determination of free amino acids. Lactic acid bacteria, however, are known for their ability to assimilate peptides (Remize et al. 2006; Ritt et al. 2008). This explanation could serve to correlate levels between different compounds (Herbert et al. 2005, 2006).

The addition of nitrogen allows rapid fermentation, limiting bacterial growth (Fig. 2). This supplement, however, acts as a precursor to enrichment. Our results demonstrate that these two opposing mechanisms result in an increase in the final concentration of biogenic amines. If it was just a matter of biogenic amines, nitrogen should be avoided. Nitrogen, however, reduces the duration of fermentation and avoids fermentation arrest. Moreover, lack of nitrogen causes yeast synthesis of higher alcohols, responsible for heavy flavours (Carrau et al. 2008; Hernández et al. 2006). Fermentation difficulties can also lead to the formation of short- and medium-chain fatty acids by the yeast. These compounds are known to inhibit bacterial growth, which may jeopardise the completion of malolactic fermentation (Alexandre et al. 2004). Excessive preventive supplementation, however, may cause repression phenomena and in some cases decrease the efficiency of fermentation (Taillandier et al. 2007). It can also result in reduced synthesis of higher alcohols, along with the formation of ethyl carbamate, or to microbial instability if the residual ammonium concentration is too high, possibly resulting in potential biogenic amine-producing bacterial. In fact, addition of nitrogen must be reasonable in order to

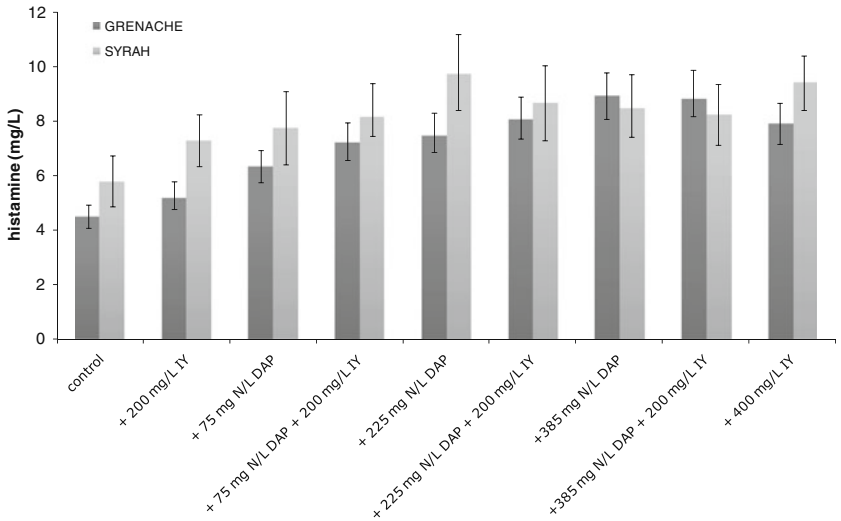

Fig. 4 histamine (mg/L) depending on grape (Syrah □, Grenache ■) and added nitrogen (*DAP* and/or inactivated yeast, *IY*). (n=2 independent experiments)

achieve a balance between fermentation and the risk of biogenic amine production.

We have shown a correlation between the amount of nitrogen added and the production of biogenic amines, without being related to the concentration of amino acid precursors. Thus, depending on the amount of nitrogen added, histamine levels increased to 10 mg/L, the limit for the export of wine to Switzerland until 2008. We can thus understand how an operation that appears benign may jeopardise the marketing of the wine produced. It has already been shown that musts supplemented with a yeast autolysate contain greater concentrations of some biogenic amines (tyramine and cadaverine)(González and Ancín 2006).

It is also important to remember that this study was conducted with a limited number of strains. The evidence is that, in these cases, only histamine was produced. As tools for characterising genetic traits associated with the production of other biogenic amines (tyramine, putrescine and cadaverine) are available (Nannelli et al. 2008), we intend to test new starters on the same base wine from this study that we could keep. We also intend to better characterise the pool of free amino acids and related oligopeptide forms for each method. Similarly, as this study raises the question of the possible presence of yeast cell precursors in wine-based products, more commercial products should be considered.

In fact, other yeast derivatives are commercialised to be used in wines to improve fermentation and wine organoleptic characteristics. Their composition is highly variable, but most of them are comprised of inactivated yeast, metabolites from yeast autolysis (such as amino acids, peptides, proteins, polysaccharides, nucleotides and fatty acids), yeast walls, vitamins and minerals. In addition, the composition of yeast autolysates may vary depending on yeast culture conditions (Guilloux-Benatier and Chassagne 2003). All of these yeast derivatives are offered by various companies, under many different names, promising very specific improvements in wine. Nevertheless, despite the growing interest in these products by the oenological sector, the information related to their composition is often unclear and can, as we have shown, impact the final biogenic amine content.

In addition, other factors such as wine pH and the characteristics of the vintage may also play a key role in amine biogenesis. This was observed between the two varieties with a particular variation in the kinetics of bacterial growth. These elements should also be taken into account in the management of malolactic starters.

In conclusion, this study has shown that some widely used oenological practices can lead to an increased risk of obtaining wines containing significant levels of biogenic amines. Thus, this study shows that the new "biogenic amine" risk management trend is often at odds with the desire to promote the quality of wine. Indeed, our work shows that, even though it reduces bacterial populations, the addition of nitrogen can lead to increased production of these compounds. Other recent studies have also shown that many actions aimed at increasing wine complexity, such as skin maceration and aging on lees, strongly influence final biogenic amine concentration in wine (Alcaide-Hidalgo et al. 2007). The fact is that these qualitative techniques cannot reasonably be completely ruled out. In this way, making a diagnosis of bacterial populations indigenous seems decisive. In case of the presence of producer bacteria, the only currently available solution for limiting the level of biogenic amines in wine is to use malolactic starters. However, the result of starters' use depends on the indigenous bacterial population level and on those characteristics of wine that can cause a bad implantation.

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