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Mead production: effect of nitrogen supplementation on growth, fermentation profile and aroma formation by yeasts in mead fermentation

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Mead is an alcoholic beverage, produced since ancient times, resulting from an alcoholic fermentation of diluted honey by yeasts. When it is produced in a traditional manner, mead producers can encounter several problems related to a lack of essential nutrients, such as available nitrogen. Thus, the aim of this study was to evaluate the effect of nitrogen addition to honey-must on the fermentation performance of two *Saccharomyces cerevisiae* wine yeasts, QA23 and ICV D47, as well as on the mead composition and production of volatile aroma compounds. A portion of honey-must was supplemented with diammonium phosphate (DAP) to achieve the nitrogen concentration required by yeast to complete alcoholic fermentation. The supplementation with DAP reduced the fermentation length to around 7 days, but not all sugars were fully consumed, suggesting that other factors could be interfering with yeast growth. For both yeasts the specific growth rate and final biomass were higher in musts supplemented with DAP. Mead final composition was similar under the two experimental conditions. Analysis of the volatile profile revealed that the concentrations of the volatile fatty acids and volatile phenols were higher in meads supplemented with DAP. The concentrations of ethyl hexanoate, ethyl octanoate and isoamyl acetate were above their perception threshold and were higher in meads supplemented with DAP, which could contribute to the enhancement of the fruity character. This study could be useful for the optimization of mead production and quality improvement.

Keywords: aromatic profile; diammonium phosphate supplementation; honey-must; *Saccharomyces cerevisiae* strains

Introduction

Mead is an alcoholic traditional drink obtained from diluted honey with the appropriate yeast inoculations. Its production represents a possible economic alternative to honey producers that intend to obtain honey derivatives with surplus value, allowing the development of a beverage possessing great commercial potential *(1)*. However, it has been reported that mead fermentation is a time-consuming process, often taking several months to accomplish, depending on the type of honey, yeast strain and honey-must composition *(2)*. Indeed, when it is produced in a traditional manner, mead producers find several problems, namely a lack of uniformity in the final product, the occurrence of sluggish and stuck fermentations and the production of undesirable flavours *(3)*. These problems could be due to several factors, including a lack of essential nutrients, among which could be a deficiency in available nitrogen.

Indeed, according to the literature, nitrogen deficiencies are one of the main causes of stuck or sluggish fermentations *(4–6)*. Under oenological conditions, in natural or synthetic grape media, it has been stated that additions of assimilable nitrogen significantly increase the fermentation rate and reduce the time required for the completion of an alcoholic fermentation *(5–8)*. Similar results have been observed with nitrogen-supplemented fermentations using different diluted honeys and different yeast strains for optimization of mead production *(9)*. However, it is necessary to take into account the legislation on limiting nitrogen addition owing to the possible formation of ethyl carbamate, a suspected carcinogen, and because excess nitrogen can cause microbial instability in wines and in other alcoholic beverages *(10)*. The mean value of 140 mg/L of amino nitrogen has been suggested as sufficient for complete fermentation of reasonably ripened grapes *(11)*, while a value six times higher has been suggested to be the optimum level *(12)*. Several studies conducted in our laboratory have shown that yeast strains require a minimum of 267 mg/L of nitrogen to attain complete alcoholic fermentation of synthetic grape juice media with 200 g/L

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of glucose *(6)*. However, industrial yeasts strains have shown substantial differences in nitrogen demands during alcoholic fermentation *(13)*, and commercial yeast strains can be categorized according to their nitrogen requirements *(14)*. However, most nitrogen additions do not take into account the different nitrogen needs of the yeast cells during wine fermentations *(5)* but are selected to obtain wines with different aroma profiles *(15)*. The nitrogen concentration regulates the formation of yeast by-products such as H_2S, fatty acids, higher alcohols and esters, among others, which affect the chemical and sensorial properties of the alcoholic beverage *(10,15,16)*.

Considering the variability of assimilable nitrogen abundance in the natural substrates used for alcoholic fermentation and the difficulties encountered in honey-must fermentations, the effects of nitrogen addition to honey-must on growth and fermentative performance, as well as on volatile aroma, were evaluated using the active dry wine yeasts *Saccharomyces cerevisiae* QA23 and ICV D47.

Material and methods

Yeast strains

Saccharomyces cerevisiae Lalvin QA23 (Lallemand, Montreal, Canada) and *S. cerevisiae* Lalvin ICV D47 (Lallemand, Montreal, Canada) were used in this study as active wine dry yeasts.

Honey

In this study, dark honey was purchased from a local beekeeper in the northeast region of Portugal. A palynological analysis was performed according to the acetolytic method previously described in detail *(17)*. This determined that the honey was multifloral and derived primarily from the pollen of *Castanea* spp. and *Erica* spp.

In accordance with the requirements established in Portuguese legislation (Decreto-Lei no. 214/2003, 18 September), the characteristics and satisfactory quality of the honey were assured through an analysis of the following parameters: moisture content, diastase index and hydroxymethylfurfural content according to Gomes *et al*. *(18)*; pH, acidity and reducing sugars (fructose and glucose) as described by Bogdanov *et al*. *(19)*; and electric conductivity and ash content as described by Sancho *et al*. *(20)*.

Preparation of honey-must for fermentation

To obtain an alcoholic beverage of approximately 11% ethanol, honey was diluted to 37% (w/v) using natural spring-water obtained from the market and mixed to homogeneity. Any insoluble materials were removed from the mixture by centrifugation (2682.8***g*** for 30 min; Eppendorf 5810 R centrifuge) to obtain a clarified honey-must. Titratable acidity was adjusted with 5 g/L of potassium tartrate (Sigma-Aldrich, St Louis, MO, USA) and pH was adjusted to 3.7 with malic acid (Merck, Darmstadt, Germany). To evaluate the effect of nitrogen addition, the nitrogen content was adjusted to 267 mg/L with diammonium phosphate (DAP; BDH Prolabo, Leuven, Belgium). In parallel, a control honey-must was prepared without DAP supplementation. The parameters °Brix, pH and assimilable nitrogen concentration were determined prior to and after the adjustments. Yeast assimilable nitrogen (YAN) was assayed by the formaldehyde method as previously described *(21)*. The honey-musts were pasteurized at 65°C for 10 min and then immediately cooled.

Fermentation conditions and monitoring

Starter cultures were prepared by rehydration of 10 g of active dry yeast in 100 mL of honey-must at 38°C according to the manufacturer's instructions to obtain 10^8 CFUs/mL. The appropriate amount of inoculum was pitched into the honey-must to obtain a pitching rate of 10^7 CFUs/mL.

All fermentations were carried out in triplicate using a previously described system *(9)*, which consisted of 250 mL flasks filled to two-thirds of their volume and fitted with a side-arm port sealed with a rubber septum for anaerobic sampling. During the alcoholic fermentation, the flasks were maintained at 25°C under permanent but moderate shaking (120 rpm/min) mimicking an industrial environment. Aseptic sampling for assessing fermentation and growth parameters was performed using a syringe-type system as previously described *(15)*. Fermentations were monitored daily using weight loss as an estimate of CO_2 production. At the same time, samples were collected and diluted appropriately for the measurement of optical density at 640 nm in a UV–visible spectrometer (Unicam Helios) and for counting CFUs in solid yeast peptone dextrose agar (20 g/L glucose, 10 g/L peptone, 5 g/L, yeast extract and 20 g/L agar) plates after incubation at 25°C for 48 h. Determination of reducing sugars was performed using the 3,5-dinitrosalicylic acid method with glucose as the standard *(22)*. At the end of alcoholic fermentation, samples were taken from all fermented media for culture dry weight determination as well as the analysis of several oenological parameters and the aroma profiles of the meads.

General oenological parameters

At the end of fermentations, oenological parameters such as pH, volatile acidity and ethanol content were determined according to standard methods *(23)*. Yeast assimilable nitrogen was determined by the formaldehyde method *(21)*.

Analysis of mead aromatic compounds

Mead produced with or without DAP addition was analysed for major volatile compounds by GC-FID and for minor volatile compounds by GC-MS. The major compounds in the samples were determined directly by the internal standard (4-nonanol) method, taking into account the relative response of the detector for each analyte. Identification was performed by a comparison of retention times with those of pure standard compounds. The minor volatile compounds were analysed after extraction with dichloromethane and quantified as 4-nonanol equivalents. Identification was made by a comparison of retention indices and mass spectra with those of pure standard compounds.

Chromatographic analysis of major volatile compounds. In a glass tube, 100 μL of an ethanolic solution with 3640 mg/L of internal standard (4-nonanol, Merck, Darmstadt, Germany) was added to 5 mL of mead. A Chrompack GC CP-9000 gas chromatograph equipped with a split/splitless injector, a flame ionization detector (FID) and a capillary column CP-Wax 57 CB (50 m × 0.25 mm; 0.2 μm film thickness) was used. The

temperatures of the injector and detector were both set to 250°C and the split ratio was 15 mL/min. The column temperature was initially held at 60°C for 5 min and then programmed to rise from 60 to 220°C at 3°C/min; finally it was maintained at 220°C for 10 min. The carrier gas was special helium 4× (Praxair) at a flow rate of 1 mL/min (125 kPa at the head of the column). The analysis was performed by the injection of 1 μL of sample. The quantification of volatile compounds, after the determination the detector response factor for each analyte, was performed with the software Star–Chromatography Workstation version 6.41 (Varian) by comparing test compound retention times with those of pure standard compounds.

Extraction of volatiles. The extraction of mead minor volatiles was performed according to the method described by Oliveira *et al. (24)*. In a 10 mL culture tube (Pyrex, reference 1636/26MP) the following were added: 8 mL of mead clarified by centrifugation, 80 μL of an ethanolic solution, 36.4 mg/L of an internal standard (4-nonanol, Merck, Darmstadt, Germany) and a magnetic stir bar (22.2 × 4.8 mm). The tube was sealed and extraction was accomplished by stirring the mead with 400 μL of dichloromethane (Merck, Darmstadt, Germany) for 15 min with a magnetic stirrer. After cooling the solutions at 0°C for 10 min, the magnetic stir bar was removed and the organic phase was separated by centrifugation (*RCF* =5118***g*** for 5 min at 4°C) and transferred into a vial with a Pasteur pipette. Finally, the aromatic extract was dried with anhydrous sodium sulphate (Merck, Darmstadt, Germany) and again transferred into a new vial.

Chromatographic analysis of minor volatile compounds. Minor volatile compounds were analysed by GC-MS using a gas chromatograph Varian 3800 with a 1079 injector and an ion-trap mass spectrometer Varian Saturn 2000. A 1 μL injection was made in splitless mode (30 s) in a Varian Factor Four VF-WAXms (30 m × 0.15 mm; 0.15 μm film thickness) column. The carrier gas was helium UltraPlus 5 × (99.9999%) at a constant flow rate of 1.3 mL/min. The detector was set to electronic impact mode with an ionization energy of 70 eV, a mass acquisition range from 35 *m/z* to 260 *m/z* and an acquisition interval of 610 ms. The oven temperature was initially 60°C for 2 min and then raised from 60 to 234°C at a rate of 3°C/min, raised from 234 to 250°C at 10°C/min and finally maintained at 250°C for 10 min. The temperature of the injector was maintained at 250°C during the analysis time and the split flow was maintained at 30 mL/min. The identification of compounds was performed using the software MS WorkStation version 6.6 (Varian) by comparing their mass spectra and retention indices with those of pure standard compounds. The minor compounds were quantified in terms of 4-nonanol equivalents.

Odour activity values

The odour activity value (OAV) was calculated for each volatile compound by dividing the concentration of each quantified compound by its perception threshold found in the literature *(25–28)*.

Statistical analysis

All fermentation experiments were performed in triplicate and results expressed as mean values and standard deviation. An analysis of variance (ANOVA) with type III sums of squares was performed using the general linear model procedure as implemented in the SPSS software, version 17.0 (SPSS Inc.). The fulfilment of the ANOVA requirements, namely the normal distribution of the residuals and the homogeneity of variance, was evaluated by means of the Shapiro–Wilks test ($n < 50$) and Levene's test, respectively. All dependent variables were analysed using a one-way ANOVA. For each strain, the main factor studied was the effect of nitrogen addition to honey-must on the physicochemical characteristics of meads, and whenever significant differences were found, an independent-samples *t*-test was performed to compare means. All statistical tests were performed at a 5% significance level.

Results and discussion

To study the effect of nitrogen addition an aliquot of honey-must was adjusted with DAP to achieve the concentration of nitrogen required by yeast to complete alcoholic fermentation. In parallel, a control fermentation was carried out without DAP addition. The honey-musts were inoculated with strains QA23 or ICV D47 at a pitching rate of 1×10^7 viable cells/mL. Yeast growth and sugar consumption were assessed during fermentation and at the end of fermentation mead composition and aroma profile were examined.

Effect of nitrogen addition on yeast growth and fermentation profile

The effect of the honey-must supplementation with DAP on growth and fermentation profiles of *S. cerevisiae* QA23 and ICV D47 are shown in Fig. 1. The supplementation of honey-must with DAP had similar effects on the fermentation profile of both strains (Fig. 1A and B). The fermentation length was reduced from 240 to 96 h, with similar amounts of reducing sugars remaining in the final fermentation. It has already been stated that the addition of assimilable nitrogen to the must resulted in an increased fermentation rate, and consequently in a significant reduction in the time required for fermentation *(4–6,9,29,30)*. The concentration of reducing sugars that remained, around 40 g/L, has been previously observed in meads *(9,31)*. However, it should be noted that these were the non-fermentable sugars such as trehalose, isomaltose, saccharose and melezitose. This is in agreement with results previously published by our group *(31)*. As expected, the ethanol content of the meads varied from 10 to 10.5%, and no correlation was found with the addition of DAP to the honey-must (results not shown). Both fermentations with DAP ended at 96 h, but the supplementation had a distinctive effect on each strain. Strain QA23 was more efficient in terms of sugar consumption than strain ICV D47, with almost all sugars consumed in the first 48 h of fermentation. Strain ICV D47 used the reducing sugars more gradually throughout the fermentation period.

Regarding the growth profile, the number of viable cells was higher in fermentations supplemented with DAP (Fig. 1C and D). The final viable biomass obtained for the control fermentations was 4.2×10^7 for *S. cerevisiae* QA23 (Fig. 1C) and 2.6×10^7 for ICV D47 (Fig. 1D), while in the supplemented fermentations the values were 6.9×10^7 and 3.5×10^7, respectively. An earlier slowdown of cell growth has been previously reported in fermentations without nitrogen supplementation *(4,9)*. Independent of DAP addition, strain QA23 presented more growth in the first 24 h, almost doubling the initial population. In particular, in the fermentation with honey-must supplemented with DAP, the

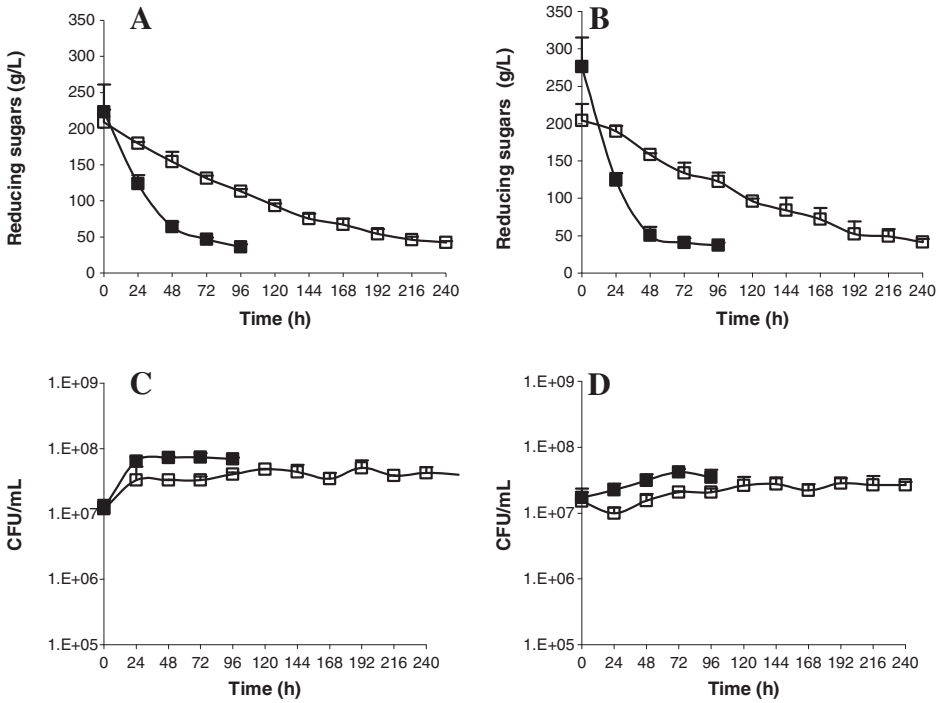

Figure 1. Fermentation and growth profiles of *Saccharomyces cerevisiae* strains in fermentations with diammonium phosphate (DAP) (■) or without DAP (□) addition: (A) fermentation profile of strain QA23; (B) fermentation profile of strain ICV D47; (C) growth profile of strain QA23; and (D) growth profile of strain ICV D47.

population nearly reached 10^8 CFUs/mL and remained constant until the end of fermentation. Strain ICV D47 showed a lower growth-rate with an almost constant population throughout the fermentation. These results indicate that nitrogen affects yeast cells in two ways: it increases biomass production and stimulates the rate of sugar utilization *(5)*. Yeast growth and fermentation rate are an exponential function of the initial nitrogen content of must, being highly sensitive to low nitrogen concentrations (less than 300 mg/L) and less responsive to higher concentrations *(10)*. Martínez-Moreno *et al. (14)* verified that the biomass yield was dependent on the amount of available nitrogen, the nature of the nitrogen source and the sugar concentration in the medium.

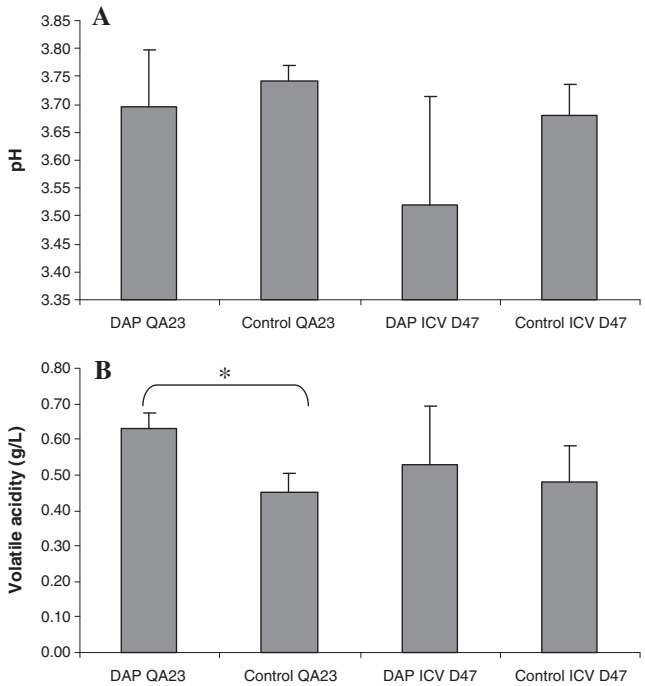

Figure 2. pH (A) and volatile acidity (g/L) (B) of meads produced by *S. cerevisiae* QA23 and ICV D47 in fermentations with DAP or without DAP addition (control). Statistical significance at $p < 0.05$, determined by a Student's *t*-test (*).

Effect of nitrogen addition on mead composition

At the end of the alcoholic fermentation, samples were collected to evaluate the final mead composition, in terms of pH, volatile acidity, and assimilable nitrogen. The monitoring of the mead pH is important, since the acetic and succinic acids produced during a mead fermentation lead to an increased non-dissociated fatty acid content, which can cause the fermentation to slow down or even stop prematurely *(32)*. There was little

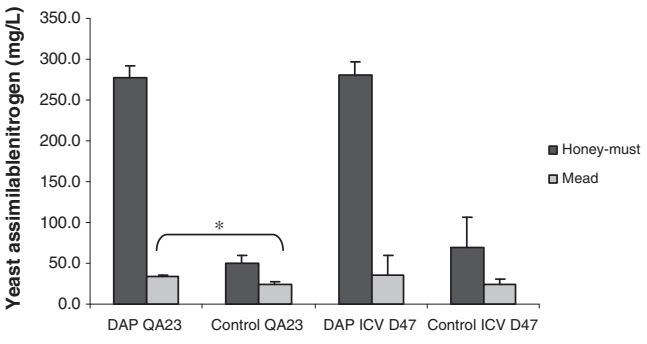

Figure 3. Concentration of yeast assimilable nitrogen in honey-must and meads produced by *S. cerevisiae* QA23 and ICV D47 in fermentations with DAP or without DAP addition (control). Statistical significance at $p < 0.05$, determined by a Student's *t*-test (*).

effect of nitrogen addition on the final pH of the mead for both strains, which varied from 3.52 to 3.74 (Fig. 2A). Ammonium ion uptake is associated with the excretion of proton ions into the medium, thereby decreasing extracellular pH *(16)*. The final pH values of meads fermented by the strain ICV D47 were slightly lower than the ones obtained with strain QA23. For both strains, the volatile acidity, expressed as g/L of acetic acid, was higher in meads obtained from honey-must supplemented with DAP (Fig. 2B). Significant differences between supplemented and non-supplemented fermentations were only obtained for QA23. These results were inconsistent with previous work on mead fermented with other strains of *S. cerevisiae* and also supplemented with different concentrations of ammonium *(9)*, suggesting that these differences could be due to different yeast nitrogen demands. Although the values obtained in all meads were in agreement with values previously reported for this alcoholic beverage *(3,9,31,32)*, irrespective of the yeast or the honey used, in the presence of DAP, the strain QA23 produced higher volatile acidity than the strain ICV D47 (0.63 and 0.53 g/L of acetic acid, respectively). Figure 3 shows the concentrations of yeast assimilable nitrogen in honey-must and meads produced for both strains in fermentations with and without DAP supplementation. Independent of DAP supplementation, at the end of the alcoholic fermentation residual assimilable nitrogen remained in all of the meads, with the lower concentration determined on the non-supplemented meads (24.50 mg/L). These values most likely correspond to the concentration of the amino acid proline, which is present in honey but is not assimilable by yeasts in the fermentation environment *(31)*. In meads supplemented with DAP and fermented by strain QA23, the final nitrogen concentration was significantly higher than in the control fermentation (Fig. 3), indicating that not all of the nitrogen was used by this strain, in agreement with previously reported results *(9)*.

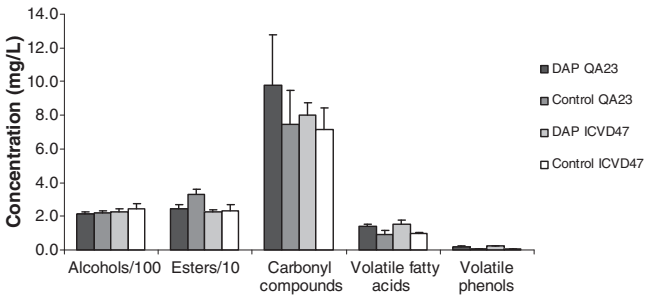

Figure 4. Profile of the volatile compounds in the meads. Fermentations conducted by *S. cerevisiae* QA23 and ICV D47 with DAP or without DAP (control) supplementation of honey-must.

Effect of nitrogen addition on mead aromatic profile

The mead aroma compounds can receive contributions from flowers, honey or fermentation. In addition to its effect on yeast growth and fermentation kinetics, YAN can regulate yeast metabolism at several levels including the formation of yeast volatile and non-volatile metabolites, which contribute to beverage flavour *(16)*. A total of 27 fermentative volatile compounds were identified and quantified in meads produced by strain QA23 and ICV D47 from the control and DAP supplemented fermentations. The compounds belonged to five different groups and Fig. 4 shows the aroma profiles of all meads. The alcohols were the major group of volatile compounds quantified in all meads, followed by the esters, carbonyl compounds, volatile fatty acids and volatile phenols. Higher alcohols and esters, produced during alcoholic fermentation, play an important role in the flavour of wine, depending on the type and concentration of the compound *(33)*. The alcohols can have a positive or a negative impact on the aroma and flavour of alcoholic beverages *(10)*. Concentrations $<$300 mg/L are desirable for the complexity of an alcoholic beverage because they confer fruity characters *(8,34,35)*. All meads presented levels of alcohols $<$300 mg/L and almost no differences were detected between the fermentation with or without DAP supplementation.

The production of esters by the yeast during fermentation can have a significant effect on fruity flavours *(35)*. The highest concentration of this group of compounds was found in fermentations without nitrogen supplementation conducted by strain QA23. In the group of carbonyl compounds, only acetaldehyde was included as quantitatively it is the most important saturated aldehyde found in wine *(8)*. Acetaldehyde production appears to be dependent not only on nitrogen supplementation but also on the strain, since the highest concentrations were

Table 1. Odour activity values (OAV) of volatile compounds with an influence on the aroma of meads fermented by *S. cerevisiae* QA23 and ICV D47 with diammonium phosphate (DAP) or without DAP (control) supplementation of honey-must

Compounds	Odour descriptor[a]	Odour threshold (μg/L) [a]	OAV			
			QA23		ICV D47	
			DAP	Control	DAP	Control
3-Methyl-1-butanol	Cheese; nail polish	30 000	4.28	4.12	4.21	4.78
Ethyl butyrate	Fruity; sweet	20	2.41	2.65	2.92	1.54
Ethyl hexanoate	Fruity; aniseed	14	8.28	5.64	12.01	6.13
Ethyl octanoate	Fruity; sweet	5	46.47	14.67	63.21	18.13
Ethyl acetate	Solvent; nail polish	12 300	1.92	2.65	1.74	1.83
Isoamyl acetate	Banana	30	15.59	3.69	18.57	4.73
Octanoic acid	Fatty; rancid	500	1.70	---	1.80	1.02
Acetaldehyde	Fresh; green leaves	500	19.61	14.90	15.99	14.29

[a]Odour descriptors and odour threshold as reported in the literature *(25–28)*.

achieved in meads supplemented with DAP and utilizing strain QA23. It has already been reported that there are differences in acetaldehyde production between yeast species and strains *(36)*. Fatty acids are a group of compounds that give unpleasant aromas and are associated with fatty, rancid and cheese-like odours *(8)*. The production of these compounds appears to be enhanced by nitrogen, since higher amounts were found in the fermentation supplemented with DAP for both strains. It has been shown that the production of medium-chain fatty acids is stimulated by high assimilable nitrogen levels *(15,16)*. Nevertheless, in all fermentations, the concentration of fatty acids was lower than the taste perception threshold. Volatile phenols are known for their contribution to off-flavours *(35)*. Although the concentration of volatile phenols was higher in meads supplemented with DAP, again the levels were below perception threshold in all meads.

In order to assess the influence of the compounds studied on overall mead aroma, OAV was calculated by dividing the concentration of each compound by its perception threshold. Only the compounds with an OAV >1 contribute individually to the mead aroma *(37)*. However, a particular compound with an OAV <1 might contribute to the aroma of a beverage, because of the additive effect of similar compounds (similar structure or odour) *(33)*. In Table 1, the volatile compounds with an OAV >1 and the odour descriptor of each compound are presented. Only eight compounds presented an OAV >1 and possibly contributed to the aroma of the meads, five of them with pleasant (fruity and fresh) aroma and three with fatty/nail polish aroma. The highest OAV was represented by the esters, mainly ethyl octanoate. The concentration of this ester increased to almost 3-fold with nitrogen addition. Previous studies on mead and on Chardonnay wine have reported similar observations *(15,16)*. Isoamyl acetate also presented a considerable OAV in meads supplemented with nitrogen, confirming that its production increases with higher carbon or nitrogen concentrations *(38)*. Alcohols were the major chemical group found in all meads, mainly owing to 3-methyl-1-butanol concentration. However, the OAV of this compound was lower and similar in all meads. Acetaldehyde was the compound with the second highest OAV in all meads, suggesting a considerable impact on mead aroma. However, little difference was found between the strains and honey-must supplementation.

In conclusion, the supplementation of honey-must with DAP brought several advantages: a reduced fermentation length to approximately 7 days and a higher yeast specific growth rate and final biomass. Although mead final composition was similar in all of the meads, the volatile acidity, total SO_2 and final assimilable nitrogen were slightly higher in the fermentations supplemented with nitrogen. The assimilable nitrogen that remained at the end of fermentations supplemented with DAP suggests that the concentration supplied fulfilled the needs/demands of both yeasts. The volatile profile revealed that the concentrations of carbonyl compounds, volatile fatty acids and volatile phenols were higher in meads supplemented with DAP. Only a few compounds would contribute to the mead aroma profile with concentrations above their perception threshold. One of the most important compounds is the ester, ethyl octanoate, which contributes to fruity character in meads.

This study, despite not being the first to evaluate the effect of nitrogen supplementation on honey-must, is still an important contribution for the optimization of mead production and for improving quality. This is the first work to evaluate the nitrogen demand of strains QA23 and ICV 47 in mead production. However further research is needed, especially to investigate if the final concentration of assimilable nitrogen is quantifying some amino acids not metabolized by yeasts, such as proline.

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Conflict of Interest

No conflict of interest declared.

References

1. Ramalhosa, E., Gomes, T., Pereira, A.P., Dias, T., and Estevinho, L.M. (2011) Mead production: Tradition versus modernity, in *Advances in Food and Nutrition Research* (Jackson, R.S. Ed.) pp. 101–118, Academic Press, Burlington, VA.
2. Navrátil, M., Šturdík, E., and Gemeiner, P. (2001) Batch and continuous mead production with pectate immobilised, ethanol-tolerant yeast, *Biotechnol. Lett. 23*, 977–982.
3. Pereira, A.P., Dias, T., Andrade, J., Ramalhosa, E., and Estevinho, L.M. (2009) Mead production: Selection and characterization assays of *Saccharomyces cerevisiae* strains, *Food Chem. Toxicol. 47*, 2057–2063.
4. Bely, M., Salmon, J.M., and Barre, P. (1994) Assimilable nitrogen addition and hexose transport system activity during enological fermentation, *J. Inst. Brew. 100*, 279–282.
5. Beltran, G., Esteve-Zarzoso, B., Rozès, N., Mas, A., and Guilllamón, J.M. (2005) Influence of the timing of nitrogen additions during synthetic grape must fermentations on fermentation kinetics and nitrogen consumption, *J. Agric. Food Chem. 53*, 996–1002.
6. Mendes-Ferreira, A., Mendes-Faia, A., and Leão, C. (2004) Growth and fermentation patterns of *Saccharomyces cerevisiae* under different ammonium concentrations and its implications in winemaking industry, *J. Appl. Microbiol. 97*, 540–545.
7. Betite, V.C., Júnior, M.M., Oliveira, J.E., and Ernandes, J.R. (2012) Very high gravity sucrose fermentation by Brazilian industrial yeast strains: Effect of nitrogen supplementation, *J. Inst. Brew. 118*, 174–178.
8. Ugliano, M. and Henschke, P.A. (2009) Yeasts and wine flavour, in *Wine Chemistry and Biochemistry* (Moreno-Arribas, M.V. and Polo, M. C. Eds.) pp. 313–392, Springer Science + Business Media, New York.
9. Mendes-Ferreira, A., Cosme, F., Barbosa, C., Falco, V., Inês, A., and Mendes-Faia, A. (2010) Optimization of honey-must preparation and alcoholic fermentation by *Saccharomyces cerevisiae* for mead production, *Int. J. Food Microbiol. 144*, 193–198.
10. Mendes-Ferreira, A., Barbosa, C., Lage, P., and Mendes-Faia, A. (2011) The impact of nitrogen on yeast fermentation and wine quality, *Cienc. Téc. Vitiv. 26*, 17–32.
11. Agenbach, W.A. (1977) A study of must nitrogen content in relation to incomplete fermentations, yeast production and fermentation activity, in *Proceedings of the South African Society of Enology and Viticulture*, pp. 66–87, South African Society of Enology and Viticulture, Cape Town.
12. Kunkee, R.E. (1991) Relationship between nitrogen content in must and sluggish fermentation, in *Proceedings International Symposium on Nitrogen in Grapes and Wine* (Rantz, J. Ed.) pp. 148–155, American Society for Enology and Viticulture, Davis, CA.
13. Manginot, C., Roustan, J.L., and Sablayrolles, J.M. (1998) Nitrogen demand of different yeast strains during alcoholic fermentation. Importance of the stationary phase, *Enz. Microb. Technol. 23*, 511–517.
14. Martínez-Moreno, R., Morales, P., Gonzales, R., Mas, A., and Beltran, G. (2012) Biomass production and alcoholic fermentation performance of *Saccharomyces cerevisiae* as a function of nitrógeno source, *FEMS Yeast Res. 12*, 477–485.
15. Mendes-Ferreira, A., Barbosa, C., Falco, V., Leão, C., and Mendes-Faia, A. (2009) The production of hydrogen sulphide and other aroma compounds by wine strains of *Saccharomyces cerevisiae* in synthetic media with different nitrogen concentrations, *J. Ind. Microbiol. Biotechnol. 36*, 571–583.

16. Torrea, D., Varela, C., Ugliano, M., Ancin-Azpilicueta, C., Francis, I.L., and Henschke, P.A. (2011) Comparison of inorganic and organic nitrogen supplementation of grape juice – Effect on volatile composition and aroma profile of a Chardonnay wine fermented with *Saccharomyces cerevisiae* yeast, *Food Chem. 127*, 1072–1083.
17. Pires, J., Estevinho, M.L., Feás, X., Cantalapiedra, J., and Iglesias, A. (2009) Pollen spectrum and physico-chemical attributes of heather (*Erica* sp.) honeys of north Portugal, *J. Sci. Food Agric. 89*, 1862–1870.
18. Gomes, S., Dias, L.G., Moreira, L.L., Rodrigues, P., and Estevinho, L. (2010) Physicochemical, microbiological and antimicrobial properties of commercial honeys from Portugal, *Food Chem. Toxicol. 48*, 544–548.
19. Bogdanov, S., Martin, P., and Lüllmann, C. (1997) Harmonised methods of the European Honey Commision, *Apidologie 28*, 1–59.
20. Sancho, M.T., Muniategui, S., Sánchez, P., Huidobro, J.F., and Simal, J. (1991) Mieles del Pais Vasco, XI: Evaluación de los distintos tipos de cenizas, *Anales Bromat. XLIII-4*, 311–324.
21. Aerny, J. (1996) Composés azotes des moûts et des vins, *Rev. Suisse Vitic. Arboric. Hortic. 28*, 161–165.
22. Miller, G.L. (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar, *Anal. Chem. 31*, 426–428.
23. OIV (2006) *Recueil des méthodes internationales d'analyse des vins et des mouts*, Organisation International de la Vigne e du Vin, Paris.
24. Oliveira, J.M.., Faria, M., Sá, F., Barros, F., and Araújo, I.M. (2006) C_6-alcohols as varietal markers for assessment of wine origin, *Anal. Chim. Acta 563*, 300–309.
25. Escudero, A., Gogorza, B., Melús, M.A., Ortín, N., Cacho, J., and Ferreira, V. (2004) Characterization of the aroma of a wine from Maccabeo. Key role played by compounds with low odor activity values, *J. Agric. Food Chem. 52*, 3516–3524.
26. Ferreira, V., López, R., and Cacho, J.F. (2000) Quantitative determination of the odorants of young red wines from different grape varieties, *J. Sci. Food Agric. 80*, 1659–1667.
27. Guth, H. (1997) Quantification and sensory studies of character impact odorants of different white wine varieties, *J. Agric. Food Chem. 45*, 3027–3032.
28. Moreno, J.A., Zea, L., Moyano, L., and Medina, M. (2005) Aroma compounds as markers of the changes in sherry wines subjected to biological ageing, *Food Control 16*, 333–338.
29. Ugliano, M., Fedrizzi, B., Siebert, T., Travis, B., Magno, F., Versini, G., and Henschke, P.A. (2009) Effect of nitrogen supplementation and *Saccharomyces* species on hydrogen sulfide and other volatile sulfur compounds in Shiraz fermentation and wine, *J. Agric. Food Chem. 57*, 4948–4955.
30. Palma, M., Madeira, S.C., Mendes-Ferreira, A., and Sá-Correia, I. (2012) Impact of assimilable nitrogen availability in glucose uptake kinetics in *Saccharomyces cerevisiae* during alcoholic fermentation, *Microb. Cell Fact. 11*, 99.
31. Pereira, A.P., Mendes-Ferreira, A., Oliveira, J.M., Estevinho, L.M., and Mendes-Faia, A. (2013) High-cell-density fermentation of *Saccharomyces cerevisiae* for the optimisation of mead production, *Food Microbiol. 33*, 114–123.
32. Sroka, P., and Tuszyński, T. (2007) Changes in organic acid contents during mead wort fermentation, *Food Chem. 104*, 1250–1257.
33. Vilanova, M., Genisheva, Z., Masa, A., and Oliveira, J.M. (2010) Correlation between volatile composition and sensory properties in Spanish Albariño wines, *Microchem. J. 95*, 240–246.
34. Mateo, J.J., Jiménez, M., Pastor, A., and Huerta, T. (2001) Yeast starter cultures affecting wine fermentation and volatiles, *Food Res. Int. 34*, 307–314.
35. Swiegers, J.H., Bartowsky, E.J., Henschke, P.A., and Pretorius, I.S. (2005) Yeast and bacterial modulation of wine aroma and flavour, *Aust. J. Grape Wine Res. 11*, 139–173.
36. Cheraiti, N., Guezenec, S., and Salmon, J.M. (2010) Very early acetaldehyde production by industrial *Saccharomyces cerevisiae* strains: A new intrinsic character, *Appl. Microbiol. Biotechnol. 86*, 693–700.
37. Vilanova, M., Genisheva, Z., Bescansa, L., Masa, A., and Oliveira, J.M. (2009) Volatile composition of wines from cvs. Blanco lexítimo, Agudelo and Serradelo (*Vitis vinifera*) grown in Betanzos (NW Spain), *J. Inst. Brew. 115*, 35–40.
38. Saerens, S.M.G., Delvaux, F., Verstrepen, K.J., van Dijck, P., Thevelein, J.M., and Delvaux, F.R. (2008) Parameters affecting ethyl ester production by *Saccharomyces cerevisiae* during fermentation, *Appl. Env. Microbiol. 74*, 454–459.