

Improvement of mead fermentation by honey-must supplementation

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Through honey's fermentation, diverse beverages can be obtained, among which is mead, an alcoholic drink with 8 to 18% of ethanol (v/v). Since honey is a matrix with a low nutrient concentration and other unfavourable growth conditions, several problems are usually encountered, namely delayed or arrested fermentations, unsatisfactory quality parameters and lack of uniformity of the final product, as well as unpleasant sensory properties. In this context, the aim of this work was to optimize mead production through honey-must supplementation with (a) salts, (b) vitamins or (c) salts + vitamins. The effects of the honey-must formulation on the fermentation kinetics, growth profile and physicochemical characteristics of final meads were evaluated. The results showed minor differences in the fermentation profile and time between fermentations with the different formulations. The growth profile was influenced more by the yeast strain than by the supplements added to the honey-must. In general, the honey-must composition did not influence meads' final characteristics, except regarding the SO₂ concentration of the meads produced using the strain QA23. In summary, the addition of salts and/or vitamins to honey-must had no positive effects on the fermentation, growth profile or characteristics of the final products. Copyright © 2015 The Institute of Brewing & Distilling

Keywords: fermentation; honey-must; mead; salts; vitamins

Introduction

Honey is a natural product with recognized biological activity, whose composition depends on the floral origin, climate, environmental and seasonal conditions, as well as on agricultural practices (1–6). Honey contains about 200 different substances, with the main constituents being carbohydrates and minor components minerals, proteins, vitamins, lipids, organic acids and amino acids (1,3,6,7).

The increasing appreciation of beehive products by consumers has boosted honey production, promoting the economic development of the beekeeping industry (8). As such, the development of honey-derived products, such as mead, especially using honey unsuitable for commercialization, is important to provide innovative alcoholic drinks to consumers and to increase beekeepers' profits (6).

Mead results from the alcoholic fermentation of diluted honey performed by yeasts, and contains between 8 and 18% ethanol (v/v). Even though this product is perhaps the oldest fermented drink known, its production, to a great extent, continues to occur empirically and has recently decreased. This is due, in some measure, to insufficient scientific progress in the field (9).

Mead's fermentation is a time-consuming process, taking from weeks to months to complete, and the quality of the final product is highly variable (9,10). Indeed, especially when produced in a homemade way, producers find several problems, namely, the lack of uniformity in the final product, slow or premature fermentations arrest, and the production of 'off-flavours' by the yeasts (11).

In the context of wine production, similar problems are usually associated with the yeast strain's inability to adapt to unfavourable growth conditions, such as limitations in nutrients, osmotic stress, ethanol toxicity and temperature shock stresses (12–14).

In mead production, little evidence is available concerning the importance of the supplementation of honey-must with nutrients (11),

DAP (15,16) or bee pollen (17) for improving the fermentation rates and the final characteristics of the beverage. Moreover, Pereira *et al.* (11) verified that mead production depends not only on the supplements added to the fermentation medium, but also on the honey used, since better results were obtained with dark honey, which has a higher mineral content and pH. Thus, the variation of honey composition must be taken into account in the addition of supplements, in order to create optimal fermentation conditions.

The correction of wort nutritional deficiencies in minerals and vitamins may reduce the stress sensitivity of yeast, improving the fermentation performance (18). Indeed, yeast cells require diverse vitamins, such as meso-inositol, pantothenic acid and biotin. In addition, the assimilation and storage of biotin influences the growth rate, being therefore essential for the success of the fermentation (19).

For this reason, the aim of this work was to investigate the effect of honey-must supplementation on mead production. The musts had added salts, vitamins or salts + vitamins and the fermentations were conducted with two active dry wine yeast strains (QA23 and IVC D47). In parallel, a control fermentation without minerals or vitamin supplementation was conducted under the same conditions. The fermentation profile and yeast growth, as well as the mead's final composition, were evaluated in order to determine the most adequate honey-must formulation for mead production.

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Material and methods

Yeast strains

Two *Saccharomyces cerevisiae* strains, Lalvin QA23 (Lallemand, Montreal, Canada) and Lalvin ICV D47 (Lallemand, Montreal, Canada), were used in this study as dry active wine yeasts. The starter cultures were rehydrated in water at 38°C according to the manufacturer's instructions and inoculated onto Yeast Peptone Dextrose agar (20 g/L glucose, 10 g/L peptone, 5 g/L yeast extract and 20 g/L agar). Incubation was carried out at 25°C for 3–5 days.

Honey

In this study, dark honey, purchased from a local beekeeper in the northeast region of Portugal, was used. A palynological analysis of the honey was performed according to the acetolytic method (20) and it was determined that this multifloral honey was derived primarily from the pollen of *Castanea* spp. and *Erica* spp. In accordance with 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.* (21); pH, acidity and reducing sugars (fructose and glucose) as described by Bogdanov *et al.* (22); and electric conductivity and ash content as described by Gomes *et al.* (21).

Preparation of honey-must for fermentation

To obtain an alcoholic beverage with approximately 11% ethanol, honey was diluted in natural spring-water obtained from the market (37% w/v), and mixed to homogeneity as previously described (15). 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. Titrable acidity was adjusted with 5 g/L of potassium tartrate (Sigma-Aldrich, St Louis, MO, USA) and the pH was adjusted with 3 g/L of malic acid (Merck, Darmstadt, Germany). The nitrogen content was adjusted to 267 mg/L with diammonium phosphate (DAP; BDH Prolabo, Leuven, Belgium). After the adjustments the honey-must was divided into four parts to perform the following fermentations:

- (1) control;
- (2) control + salts (14 g/L dipotassium phosphate, 1.23 g/L magnesium sulphate and 0.44 g/L calcium chloride);
- (3) control + vitamins (100 mg/L inositol, 2 mg/L pyridoxine, 2 mg/L nicotinic acid, 1 mg/L calcium pantothenate, 0.5 mg/L thiamine, 0.2 mg/L riboflavin and 0.125 mg/L biotin);
- (4) control + salts + vitamins.

The parameters °Brix (Optic Iyemen System, ABBE Refractometer), pH (Five Easy FE20, Mettler-Toledo), titratable acidity and assimilable nitrogen concentration were determined prior to and after the adjustments. Titratable acidity was determined according to standard methods (23). Yeast assimilable nitrogen (YAN) was determined by the formaldehyde method as previously described (24). The honey-musts were pasteurized at 65°C for 10 min and then immediately cooled.

Fermentation conditions and monitoring

For all experiments, starter culture was prepared by pre-growing the yeasts overnight in 100 mL flasks, containing 70 mL of Yeast Nitrogen Base (without amino acids and without ammonium sulphate) with 10% glucose and 1 g/L DAP. Incubation was performed at 25°C in an orbital shaker at 120 rpm. The appropriate amount of inoculum was pitched into the honey-musts to obtain an initial population of 10^5 CFUs/mL.

All fermentations were carried out in triplicate, using a previously described system (15) that 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. The flasks were maintained during alcoholic fermentation at 25°C under permanent, but moderate shaking (120 rpm), mimicking an industrial environment. Aseptic sampling for assessing fermentation and growth parameters was performed using a syringe-type system as previously described (25). Fermentations were monitored daily by weight loss as an estimate of CO₂ production. At the same time, samples were collected and appropriately diluted for the measurement of their optical density at 640 nm in a UV-visible spectrometer (Unicam Helios) and for counting the CFUs on the 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. At the end of alcoholic fermentation, samples were taken from all fermented media for a culture dry weight determination, as well as for the analysis of several oenological parameters of the meads.

Analyses performed at the end of fermentation

The culture dry weight was determined using triplicate samples of 14 mL, centrifuged in pre-weighed tubes at 3890.1 g for 10 min, washed twice with sterile deionized water, dried for 24 h at 100°C and stored in a desiccator before weighing. The oenological parameters such as total sulphur dioxide (SO₂), pH, titratable acidity, volatile acidity and ethanol content were determined according to standard methods (23), and YAN was determined by the formaldehyde method (24). Determinations of reducing sugars were performed using the 3,5-dinitrosalicylic acid method with glucose as the standard.

Statistical analysis

All the 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. The main factor studied was the effect of honey-must supplementation on the physicochemical characteristics of meads and if a significant effect was found, the means were compared using Tukey's honestly significant difference multiple comparison test. All statistical tests were performed at a 5% significance level.

Results and discussion

In order to optimize mead production, the best honey-must formulation selected from a previous study of our research group

(15) was supplemented with salts, vitamins or salts + vitamins. In parallel, a control fermentation without supplementation was conducted. The honey-musts were inoculated with strains QA23 or ICV D47 to obtain an initial population of 1×10^5 CFUs/mL and yeast growth, fermentation profile and mead composition were evaluated.

Effect of honey-must supplementation on fermentation profile and on yeast growth

The effect of supplementation of honey-must on the fermentation profiles of *S. cerevisiae* QA23 and ICV D47 is presented in Fig. 1. The fermentation profile determined by the weight loss, as an estimate of CO_2 production, showed almost no differences between the fermentations with different honey-must supplementations or between the two *S. cerevisiae* strains. Even though the fermentations were conducted during 288 h, after 144–168 h, almost no additional weight loss was observed, suggesting that the fermentations had already ended.

The effect of supplementation of honey-must with minerals and/or vitamins on the growth of yeasts QA23 and ICV D47 is displayed in Fig. 2. The honey-must supplementation had a distinct effect on the growth of yeasts, with the differences between fermentations more obvious for strain QA23 than for strain ICV D47. From all fermentations performed by strain QA23, with an initial population of 10^5 CFUs/mL, the control fermentation was the one that presented a slight lag phase until 24 h. In the other fermentations with supplemented honey-must, the lag phase was almost non-existent. In the fermentation supplemented with salts, at 24 h the strain was already entering/reaching the stationary phase. Although there were differences observed between the fermentation until 48 h, after that time the growth behaviour of strain QA23 was similar in all fermentations and the population almost reached

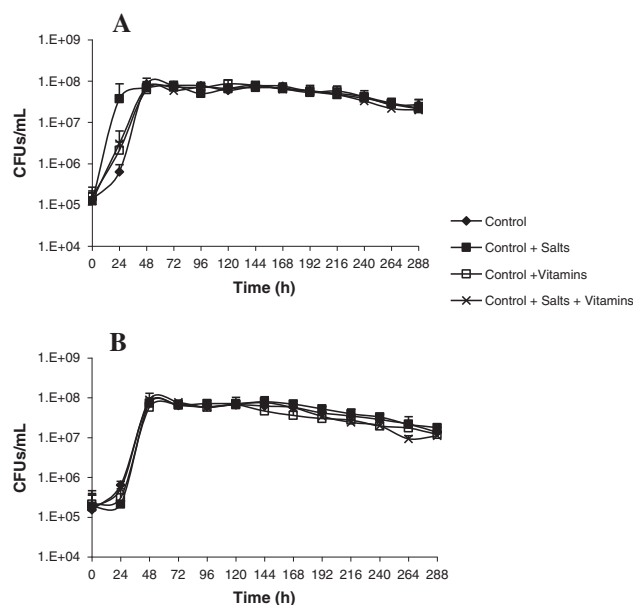


Figure 2. Growth profiles of *S. cerevisiae* QA23 (A) and ICV D47 (B) in control fermentation and fermentations with honey-must supplemented with salts, vitamins or salts + vitamins.

10^8 CFUs/mL. The growth profile of strain ICV D47 (Fig. 2B) was markedly different from the strain QA23 until 48 h of fermentation. Independently of the honey-must composition, the stationary phase of strain ICV D47 started at 48 h of fermentation. The presence of salts and vitamins increased the adaption phase of the yeast to the medium, which lasted up to 24 h. The combination of salts + vitamins in the medium reduced slightly the duration of that phase, but it was almost identical to the control fermentation. The population after 48 h of fermentation reached $7\text{--}8 \times 10^7$ CFUs/mL, and was slightly lower in the fermentations with vitamins and salts + vitamins. For both strains and in all fermentations the population remained constant between 48 and 168 h, and then decreased slightly up to 288 h, indicating, as already suggested with the weight loss (Fig. 1), that fermentations had ended at 144–168 h. Nevertheless, it would be necessary to confirm this by determining the reducing sugar consumption throughout the fermentation.

Specific nutrients, such as nitrogen, minerals or vitamins, are required to obtain rapid fermentation and high ethanol levels (19). The minerals, magnesium, calcium and zinc, influence the rate of sugar conversion and are required as cofactors for several metabolic pathways (26). Also, deficiencies in vitamins, especially thiamine and biotin, have been identified as being potentially responsible for fermentation problems, such as slow yeast growth (19,27). However, the supplementation of honey-must with vitamins or salts did not contribute significantly to enhancing the fermentation and yeast performance. These results thus indicate that the yeast's requirement for vitamins and minerals were fulfilled by the honey. The different trace and mineral element concentrations in honey depend on its botanical and geological origin (28,29), and dark honeys have a higher mineral content (0.2%) than light honeys (0.04%) (2,30). In heather honeys (*Erica* sp.) potassium, calcium and phosphorus are the minerals present at the highest levels, with potassium quantitatively being the most important mineral, possible accounting for 76% of the total mineral content (29,30). The vitamin content in honey is generally low, and includes phyllochinon (K), thiamine (B1), riboflavin (B2), pyridoxine (B6),

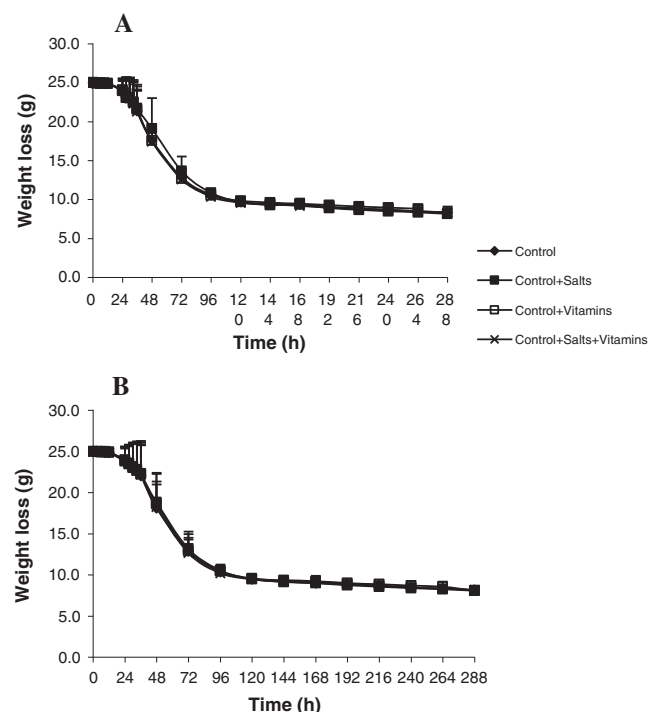


Figure 1. Fermentation profiles of *Saccharomyces cerevisiae* QA23 (A) and ICV D47 (B) in control fermentation and fermentations with honey-must supplemented with salts, vitamins or salts + vitamins.

niacin, pantothenic acid and ascorbic acid (28,31). In conclusion, the dark honey composition in terms of salts and vitamins is not a limiting factor of alcoholic fermentation and the honey appears to provide these essential compounds/nutrients for the fermentation.

Effect of honey-must supplementation on mead composition

At the end of the alcoholic fermentation, samples were taken to evaluate the mead's final composition. The parameters determined prior to fermentation in honey-musts and in the final meads, such as pH, volatile acidity, titratable acidity, final assimilable nitrogen, total SO₂ and ethanol, for strains QA23 and ICV D47 are presented in Table 1.

The low pH and the poor buffer capacity of honey could lead to a decrease in pH during the fermentation (8). The drop in pH can affect the fermentation efficiency of the strain, so the addition of a basic buffer can help by holding the pH between 3.7 and 4.0 throughout the fermentation (32). Although the pH was slightly higher in the honey-musts supplemented with salts, probably owing to the buffer capacity associated with phosphates, no significant differences were observed between the different musts. Independently of the strain, the decrease in pH during fermentation was verified in all fermentations. Even so, no significant differences were observed in final mead between fermentations with different supplementations.

The volatile acidity of meads was mainly due to the production of acetic acid by the yeast during fermentation. This acid, in an alcoholic fermentation, is produced by *S. cerevisiae* in levels that range from 0.3 to 0.8 g/L, although its formation is highly undesirable (33). The volatile acidity in all meads varied between 0.53 and 0.67 g/L, and these were similar to values previously reported in

mead (11,15,17,34–36). In general, the higher amounts of acetic acid were found in meads produced by strain QA23. Indeed, according to the information provided by the yeast producer (www.lallemand.com), strain QA23 is a slightly higher producer of volatile acidity (0.25 g/L) than strain ICV D47 (0.2 g/L).

The titratable acidity increased during fermentation from 4 g/L in the honey-must to 6.7 – 7.6 g/L, in the final meads. Increases in titratable acidity, of the order of 2–3 g/L, during the fermentation of mead has previously been reported (15,17,36). The increase in acidity is caused mainly by the synthesis of acetic and succinic acids by yeasts (36). These organic acids were probably responsible for the pH reduction during fermentation. As already verified with volatile acidity, the two strains produced different amounts of titratable acidity. As expected, based on acetic acid concentration, the titratable acidity of the meads produced by strain QA23, independent of the supplementation, was higher (above 7 g/L) than that of the meads fermented by strain ICV D47 (between 6.7 and 7.1 g/L). However, for both strains slightly lower concentrations of titratable acidity were found in the meads supplemented with vitamins.

Since honey is a poor source of nitrogen, in mead production nitrogen supplementation is a widely accepted practice to promote complete and rapid fermentation (15,32). Independent of the strain or the honey-must formulation, at the end of all fermentations a concentration of residual nitrogen, between 30 and 40 mg/L, remained in all meads. Mendes-Ferreira *et al.* (15) found similar amounts of nitrogen in mead produced with the same formulation of must as in our fermentation control. The concentration of residual nitrogen may correspond to the quantification of the amino acid proline, which is not assimilable by the yeasts. This compound represents 50–85% of the total nitrogen content of honey (2).

Table 1. Physicochemical characteristics of honey-must and meads produced by *Saccharomyces cerevisiae* QA23 and ICV D47 in control fermentation and fermentations supplemented with salts, vitamins or salts + vitamins

	Control	Control + salts	Control + vitamins	Control + salts + vitamins
<i>Honey-musts</i>				
pH	3.67 ± 0.06	3.77 ± 0.06	3.67 ± 0.04	3.78 ± 0.04
° Brix (%)	23.20 ± 0.26	23.40 ± 0.26	23.30 ± 0.20	23.23 ± 0.12
Titratable acidity _{tartaric acid} (g/L)	4.28 ± 0.24	4.13 ± 0.33	4.19 ± 0.26	4.03 ± 0.38
Initial nitrogen _{YAN} (mg/L)	263.67 ± 4.04	268.33 ± 5.35	269.50 ± 7.00	268.33 ± 11.25
<i>Meads produced by strain QA23</i>				
pH	3.61 ± 0.13	3.64 ± 0.13	3.58 ± 0.12	3.64 ± 0.11
Volatile acidity _{acetic acid} (g/L)	0.63 ± 0.11	0.67 ± 0.08	0.53 ± 0.21	0.60 ± 0.00
Titratable acidity _{tartaric acid} (g/L)	7.53 ± 0.15	7.57 ± 0.16	7.09 ± 0.16	7.29 ± 0.35
Final nitrogen _{YAN} (mg/L)	31.50 ± 3.50	39.67 ± 2.02	36.17 ± 7.29	38.50 ± 0.00
Total SO ₂ (mg/L)	13.23 ± 1.96 ^a	19.21 ± 3.39 ^{ab}	14.51 ± 1.96 ^a	22.19 ± 1.95 ^b
Ethanol (% vol)	10.33 ± 0.70	10.93 ± 0.12	10.80 ± 0.35	10.67 ± 0.23
Reducing sugars (g/L)	21.98 ± 1.09	22.10 ± 1.09	23.59 ± 2.03	21.64 ± 1.46
<i>Meads produced by strain ICV D47</i>				
pH	3.55 ± 0.13	3.66 ± 0.11	3.62 ± 0.12	3.68 ± 0.14
Volatile acidity _{acetic acid} (g/L)	0.57 ± 0.03	0.55 ± 0.05	0.60 ± 0.06	0.56 ± 0.02
Titratable acidity _{tartaric acid} (g/L)	7.06 ± 0.52	6.69 ± 0.30	6.69 ± 0.40	6.74 ± 0.41
Final nitrogen _{YAN} (mg/L)	37.33 ± 7.29	37.33 ± 14.57	32.67 ± 5.35	35.00 ± 7.00
Total SO ₂ (mg/L)	14.51 ± 0.74	14.93 ± 2.66	14.95 ± 1.96	15.79 ± 2.66
Ethanol (% vol)	10.60 ± 0.40	10.83 ± 0.40	10.93 ± 0.23	11.13 ± 0.12
Reducing sugars (g/L)	23.20 ± 2.81	23.18 ± 2.07	23.89 ± 0.52	23.56 ± 1.50

^{a, b}Indicates a significant difference within a line, $p < 0.05$. Lack of a superscript indicates no significant difference, $p > 0.05$.

Concerning the concentration of SO₂, the strains showed different behaviours in its production during the fermentations. Although no SO₂ was added to the honey-must, its concentration was detected in all of the meads at the end of fermentations. Yeasts can produce <10 mg/L SO₂ during fermentation, but in certain cases production can exceed 30 mg/L (37). For strain ICV D47, no significant differences were observed between the fermentations, with the amount of SO₂ ranging from 14.5 to 15.8 mg/L in the final meads. However, for strain QA23, the concentration of SO₂ in meads supplemented with salts and salts + vitamins was significantly higher. The production of SO₂ can be affected by fermentation conditions such as the nutritional composition of the medium (38) and the choice of yeast strains (39).

As expected, ethanol concentration ranged between 10.33 and 11.13% (vol.) and almost no differences were detected between strains. For both strains, the meads of the control fermentation presented a slightly lower ethanol content. In all fermentations, independent of the strain and honey-must supplementation, reducing sugars remained a concentration of around 21–24 g/L. These sugars were probably the non-fermentable sugars present in the honey and quantified by the method. Residual sugars were also determined by GC-MS and the results confirmed the presence of trehalose, isomaltose, saccharose and melezitose (16,34).

Conclusions

The present study's aim was to evaluate the potential of the nutritive enhancement of honey-must within the scope of the improvement of mead's fermentation performance. It was observed that, in the first hours of fermentation, the honey-must composition had a distinct effect on the growth of each strain, but this effect was diluted throughout the fermentation. The supplementation with vitamins or salts did not reduce the fermentation length, nor did it improve the quality of the final meads. No improvement in fermentation and yeast performance was observed after the honey-must supplementation with salts or vitamins, suggesting that the dark honey composition was able to provide all of the essential compounds for fermentation.

Even though further studies are needed, the results suggest that reduced yeast fermentative ability and the consequent increased risk of difficult fermentations are due to factors other than a low availability of vitamins and salts in the honey-musts.

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Conflict of interest

No conflict of interest declared.

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