Universidade de Trás-os-Montes e Alto Douro

Optimization of mead production: design of different strategies for improvement of alcoholic fermentation

Tese de Doutoramento em
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Communications

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ABSTRACT

Mead is a traditional drink that contains 8 % to 18 % (v/v) of ethanol, resulting from the alcoholic fermentation of diluted honey by yeasts. Honey is a matrix with a low nutrient concentration and other unfavourable growth conditions, so several problems are usually encountered in mead production, namely delayed or arrested fermentations, unsatisfactory quality parameters, as well as unpleasant sensory properties. Also, mead fermentation is a time-consuming process and the quality of the final product is highly variable.

In this context, the global objective of this PhD research project was the optimization of growth and fermentative performance of yeast leading to the maximization of mead quality. To achieve this purpose, two wine yeast strains of *Saccharomyces cerevisiae*, QA23 and ICV D47 were selected to conduct several fermentation trials.

Initially, it was evaluated the potential of the nutritive enrichment of honey-must to improve mead fermentation. The must was supplemented with salts, vitamins or a mixture of both, although those additions had no positive effects on yeast growth, fermentation profile or on the characteristics of the mead.

In this line further strategies have been tested in an attempt to optimize the fermentative process, including the use of high cell density or cell immobilization. The increasing of inoculum size resulted in a reduction of fermentation length, although no additional positive effects where verified in yeasts net growth. At the lowest innoculum size, minor differences were detected in the growth kinetics between the two strains.

In order to assess the most effective alginate concentration for immobilization, the two yeast strains were entrapped in 2 or 4% (w/v) alginate beads, although neither of the concentrations was able to prevent the cell leakage from the beads. So, the immobilization of yeast cells on single-layer Ca-alginate or double-layer alginate-chitosan was applied to mead production. Immobilization had no adverse effect on cell viability, since minor differences were found on fermentation kinetics between fermentations conducted with free or encapsulated cells. Also, the double-layer alginate-chitosan had no advantage compared with the single-layer Ca-alginate, as the number of free cells in the medium, resulting from cell leakage, was similar.

In addition to the studies of yeast growth kinetics and fermentative performance, all fermentations have also been screened for the production of aroma volatile compounds and for the physicochemical characteristics of meads. Identification and quantification of volatile

compounds was performed by GC-FID and GC-MS in all meads at the end of alcoholic fermentation. The results obtained showed that mead final aroma composition was dependent on the inoculum size: the formation of the volatile compounds in concentrations above their detection thresholds was particularly pronounced at low inoculum sizes. Immobilized cells produced meads with higher concentrations of compounds with fruity characteristics and of undesirable compounds. The esters isoamyl acetate, ethyl octanoate and ethyl hexanoate and acetaldehyde were the major powerful odorants found in all mead, although their concentrations varied according to the inoculum size and cell immobilization. In general, the oenological quality of meads was not influenced by the inoculum size or immobilizations, except for the volatile acidity. Mead obtained with entrapped yeast cells or with higher inoculum size presented more acetic acid.

Finally, fermentations were conducted in higher volumes to evaluate a possible correlation between aroma compound formation and the sensory attributes of mead. The most pleasant aroma compounds formation was detected in mead fermented by non-immobilized yeast cells. Sensory analysis corroborates this observation, revealing that the most pleasant aroma descriptors were correlated with mead obtained with yeast free cells, independently of the strain.

In sum, the conditions that improved the fermentation and growth performance were not necessarily associated with high quality mead.

Keywords: aroma volatile compounds; honey-must supplementation; inoculum size; mead; sensory analysis; yeasts immobilization.

RESUMO

O hidromel é uma bebida tradicional que contém 8% a 18% (v/v) de etanol e resulta da fermentação alcoólica de mel diluído, realizada por leveduras. Como o mel é uma matriz com uma baixa concentração de nutrientes e outras condições desfavoráveis de crescimento, normalmente são vários os problemas encontrados na produção de hidromel, nomeadamente, paragens ou amuos de fermentação, parâmetros insatisfatórios de qualidade, bem como propriedades sensoriais desagradáveis. Além disso, a fermentação do hidromel é um processo moroso e a qualidade do produto final é muito variável.

Neste contexto, o objectivo global deste projecto de doutoramento foi a optimização do crescimento e desempenho fermentativo da levedura para conduzir à maximização da qualidade do hidromel. Para atingir esta finalidade foram seleccionadas duas estirpes da levedura *Saccharomyces cerevisiae*, QA23 e ICV D47, para realizar vários ensaios de fermentação.

Inicialmente, para melhorar a fermentação do hidromel, avaliou-se o potencial do enriquecimento nutritivo do mosto-mel. O mosto foi suplementado com sais, vitaminas ou uma mistura de ambos, embora essas adições não tenham tido efeitos positivos no crescimento de leveduras, no perfil de fermentação ou nas características do hidromel.

Na tentativa de optimizar o processo fermentativo, outras estratégias foram testadas, incluindo a utilização de elevadas densidades celulares ou a imobilização das células. O aumento da quantidade de inóculo resultou numa redução do tempo de fermentação, embora não se tenham verificado efeitos positivos adicionais no crescimento das leveduras. Com a menor quantidade de inóculo, detectaram-se pequenas diferenças na cinética de crescimento entre as duas estirpes.

Para avaliar a concentração de alginato mais eficaz para a imobilização, as duas estirpes de levedura foram encapsuladas em esferas com 2 ou 4% (p/v) de alginato, embora nenhuma das concentrações tenha sido capaz de evitar a saída das células das esferas para o meio. Assim, a imobilização das células de levedura em camada simples de Ca-alginato ou em camada dupla de alginato-quitosano foi aplicada na produção de hidromel. A imobilização não teve qualquer efeito adverso na viabilidade celular, uma vez que foram observadas diferenças mínimas na cinética de fermentação entre as fermentações conduzidas com células livres ou encapsuladas. Além disso, a imobilização em camada dupla de alginato-quitosano não apresentou nenhuma vantagem comparativamente com a camada simples de Ca-alginato,

uma vez que o número de células livres no meio, resultante da saída das células das esferas, foi semelhante.

Adicionalmente aos estudos de cinética de crescimento das leveduras e de desempenho fermentativo, pesquisaram-se, em todas as fermentações, tanto a produção de compostos voláteis aromáticos como as características físico-químicas dos hidroméis. No final da fermentação alcoólica procedeu-se à identificação e quantificação dos compostos voláteis dos hidroméis por GC-FID e GC-MS. Os resultados obtidos mostraram que a composição aromática final do hidromel dependeu da concentração do inóculo: a formação de compostos voláteis em concentrações acima dos seus limites de detecção foi particularmente pronunciada com concentrações baixas de inóculo. As células imobilizadas produziram hidroméis com concentrações mais elevadas de compostos com características frutadas e de compostos indesejáveis. Os ésteres, acetato de isoamilo, octanoato de etilo e hexanoato de etilo e o acetaldeído foram os principais odorantes encontrados em todos os hidroméis, embora as concentrações tenham variado de acordo com a concentração do inóculo e a imobilização das células. Em geral, a qualidade enológica de hidroméis, excepto a acidez volátil, não foi influenciada pela concentração do inóculo ou imobilização. O hidromel obtido com células de levedura imobilizadas ou com maiores concentrações de inóculo apresentou mais ácido acético.

Finalmente, as fermentações foram realizadas em volumes mais elevados para avaliar uma possível correlação entre a formação dos compostos aromáticos e os atributos sensoriais do hidromel. A formação dos compostos de aromáticos mais agradáveis foi detectada no hidromel fermentado por células de levedura não imobilizadas. A análise sensorial também corroborou esta observação, revelando que os descritores de aroma mais agradáveis estavam correlacionados com o hidromel obtido com células de levedura livres, independentemente da estirpe.

Resumindo, as condições que melhoraram os desempenhos fermentativo e de crescimento não resultaram, necessariamente, em hidromel de alta qualidade.

Palavras-chave: suplementação do mosto-mel; concentração do inóculo; hidromel; análise sensorial; compostos voláteis aromáticos; imobilização de leveduras.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
CFU	Colony-forming unit
DAP	Diammonium phosphate
DNS	3,5-dinitrosalicylic acid
GC	Gas chromatography
GC-FID	Gas chromatography with flame ionisation detector
GC-MS	Gas chromatography mass spectrometry
GLM	General Linear Model
HMF	Hydroxymethylfurfural
HPLC	High performance liquid chromatography
ISO	International Organisation for Standardization
MCFA	Medium chain fatty acids
OAV	Odour activity value
OIV	Organisation Internationale de la Vigne et du Vin
PC	Principal component
PCA	Principal component analysis
PR	Pitching rate
PVA	Polyvinyl alcohol
RCF	Relative centrifugal force
rpm	Revolutions per minute
SPSS	Statistical Package for the Social Sciences
UV	Ultraviolet light
VFA	Volatile fatty acids
YAN	Yeast assimilable nitrogen
YPD	Yeast peptone dextrose

CHAPTER 1

General Introduction

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Introduction to mead

Definition, historical aspects of mead and perspectives

Mead is a traditional alcoholic beverage containing an alcoholic strength, by volume, between 8 % and 18 %, resulting from the alcoholic fermentation of diluted honey by yeasts. It is a popular beverage in Eastern Europe (Poland, Slovenia) and in the Baltic states, being also widely consumed in England, Germany, and, especially, in the African countries, among which Ethiopia and South Africa. In Portugal mead is still homemade, produced according to the traditional and empirical procedures. This alcoholic beverage is recognized as the oldest consumed by man, perhaps even before wine and probably the precursor of beer. It has a long heritage of use for over 5000 years, even though the available archaeological evidence for its production dates back to 7000 BC. The first batch of mead probably occurred when it rained into someone's open a pot of honey and the wild yeast did the rest (Kime and Morse, 1998). Pottery vessels containing mixture of mead, rice and other fruits with organic compounds of fermentation have been found in northern China; the first known description was found in Rigveda and dates back to 1700 to 1100 BC (Gupta and Sharma, 2009). The long tradition of mead consumption led to the coining of the term *honeymoon*, since besides being drunk in great quantities at weddings, the newlyweds usually had the practice of drinking mead for one month (a moon) after the ceremony, with the belief that a child would be born nine months later (National Honey Board, 2001).

Although in past its use was widespread, the development of civilizations and agricultural resources triggered the replacement of mead by other beverages, like wine and beer, in many areas of the world. In northern Europe, where vines are not cultivated, mead consumption was quite popular until wine was imported at a low cost from the southern regions. In the last few years there has been a huge spike in demand, after the drink became fashionable in America. The American Mead Makers Association, an organization dedicated to promoting mead and bringing together mead makers, lists almost 240 mead-brewers in the United States and 40 in the rest of the world.

Mead styles

Mead is an alcoholic beverage made by fermenting a mixture of honey and water. Depending on the proportion to which honey is diluted, different types of mead are obtained at 1:0.5, 1:1, 1:2 or 1:3 (honey:water). Worts that contain high concentration of sugar (1:0.5 or 1:1) are prepared in fed-batch, successively adding appropriate portions of honey to avoid premature fermentation arrest, due to excessive osmotic pressure (Sroka and Tuszyński, 2007). A weak or watered mead is called hydromel and sack mead is a sweeter mead due to the addition of honey (National Honey Board, 2001). Using a terminology similar to the used in wine, mead styles are classified as dry, semi-sweet or sweet, according to its final sugar concentration (Morales et al., 2013).

In order to enhance its character and complexity, a variety of fruits, vegetables, herbs or spices (ginger, cardamom, cloves, thyme, rosemary, bay leaves, sage, parsley, fennel, cinnamon, nutmeg, lemon or orange peels, among others) may be added to during or after fermentation. Traditional mead or show mead is made either using honey from a particular flower source or a multifloral honey. Show mead is produced only by honey's fermentation; nutrients and additives are tolerable but additional spices, fruits or herbs are not allowed. Regarding traditional mead, small amounts of spices, fruits or herbs are permitted without ever overpowering the honey flavour or aroma (McConnell and Schramm, 1995).

According to the American Mead Makers Association (www.meadmakers.org) there are several mead styles depending on local traditions and specific recipes. Pyments, cysers and melomels are types of mead that include the addition of fruit or fruit juices. Pyment is a fermented beverage made from a mixture of grape juice and honey or from a blend of grape wine and mead after fermentation. It has a distinct grape wine character, manifested in acidity, tannin and other grape characteristics, but the honey character should balance the fruity flavours. Cysers or apple honey cider are made from a mixture of honey and apple juice or cider without additional water. This beverage has an apple distinct character with a pronounced honey aroma, sweet and similar to a sherry. Concerning melomels, these are meads that contain one or a blend of fruits which contribute with subtle acidic notes to intense, instantly recognizable fruit flavours. Metheglin is mead made with spices and/or herbs, and Rhodomel is made from honey and rose petals. Other alcoholic beverage that can be made from honey is Braggot, a type of beer made with a mixture of honey and malt, characterized by an aroma of honey and malt, with some bitterness due to hop. Also, brandies and spirits can be produced from distilling mead. In addition, a sparkling beverage with high amounts of

carbon dioxide resulting from a second natural fermentation either in bottle or in tanks may be produced (National Honey Board, 2001).

Mead products derivatives

Honey is produced practically in the whole world, 90 % of which is consumed as table honey and 10 % is distributed among food, cosmetic and pharmaceutical industry. Different new products may be produced from mead, among which vinegar. In fact, by performing the acetic fermentation of mead, Ilha et al. (2000), obtained approximately 5 L of honey vinegar with 90 g/L of acetic acid, using 1 kg of bee honey as raw material. This honey-vinegar showed an acceptability index over 70 % for appearance, colour, odour and flavour, indicating its good consumer acceptability. In South Africa it is possible to find in a meadery, "Makana Meadery, www.iqhilika.co.za", honey mead mustard made by mixing whole grain black mustard with freshly ground yellow mustard and mead vinegar, honey and salt, resulting in a product with a complex flavour.

Characterization of honey

Honey is "the natural sweet substance produced by honey bees from the nectar of plants (blossom honey or nectar honey) or from secretions of living parts of plants or excretions of plant sucking insects on the living parts of plants (honeydew honey), which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in the honey comb to ripen and mature" (Codex Alimentarius, 2001). For a long time in human history, it was an important source of carbohydrate and the only largely available natural sweetener (Bogdanov et al., 2008; Feás et al., 2010). Besides its nutritional properties, honey is one of the products most referred in old traditional medicine, due its therapeutic potential, in treating respiratory and gastrointestinal illnesses, in healing wounds and burns, as an antimicrobial agent, among other biological proprieties (Al-Mamary et al., 2002; Mulu et al., 2004).

According to its botanical origin, honey can be classified in monofloral or multifloral in which the bees forage predominantly on one type of plant or several botanical species, respectively (Alvarez-Suarez et al., 2014).

Honey is mainly composed by carbohydrates, lesser amounts of water and minor components (Bogdanov, 2011) such as, minerals, proteins, vitamins, lipids, organic acids, amino acids, phenolic compounds, enzymes and other phytochemicals (Bertoncelj et al., 2007; Buba et al., 2013; Finola et al., 2007). Nevertheless, honey composition is rather variable and dependent on floral source, climate, environmental and seasonal conditions as well as handling and processing practices of its production (Al-Mamary et al., 2002; Alvarez-Suarez et al., 2014; Anklam, 1998; Arráez-Román et al., 2006; Azeredo et al., 2003; Baltrušaitytė et al., 2007; Chua et al., 2015; Kirs et al., 2011; Küçük et al, 2007).

Carbohydrates

Carbohydrates account for about 95 % to 99 % of dry matter (Bogdanov, 2011; Olaitan et al., 2007). Fructose (38.2 % as mean value) and glucose (mean value of 31.3 %), are the major carbohydrates in honey followed by sucrose (mean value of 0.7 %) (Bogdanov et al., 2008; Bogdanov, 2011). Additionally, other 25 different oligosaccharides have been detected (Bogdanov et al., 2008; Bogdanov, 2011) which include maltose, isomaltose, trehalose, turanose; trisaccharides, erlose, raffinose and melezitose; and trace amounts of tetra and pentasaccharides, among others (Anklam, 1998; Bogdanov et al., 2008) According to Codex Alimentarius (2001), the minimum concentration of the reducing sugars, glucose and fructose, is 60 % (w/w). The ratio of fructose to glucose is highly dependent on the nectar source (Anklam, 1998) and is usually 1.2/1 (de Rodríguez et al., 2004). The concentration of these sugars influences the sweetness and texture of honey: fructose is sweeter than glucose and honeys with higher ratios fructose/glucose remain liquid for longer periods since glucose is less water soluble than fructose (de Rodríguez et al., 2004; Finola et al., 2007).

Water

Water is the second most important component of honey, ranging between 15 % and 20 %, with an average value of 17.2 % (Bogdanov et al., 2008). The water content of honey depends on several factors: climate conditions, degree of maturity of the hive, and treatments applied during nectar and honey collection and storage (Finola et al., 2007; Olaitan et al., 2007). This parameter will influence its physical properties such as the viscosity (Olaitan et al., 2007). Honey with a high water content usually presents preservation and storage problems since increases the probability of the product fermentation (Olaitan et al., 2007). In

fact, low water content contributes to the stability of honey, preventing fermentation and crystallization during storage (Küçük et al., 2007).

Minerals

Minerals come from the soil and plants and are present in small amounts ranging from 0.04 %, in the clear honeys, to 0.2 %, in some dark honeys (Anklam, 1998; Fernández-Torres et al., 2005). In addition, other elements may be added during the processes of centrifugation and storage (Freitas et al., 2006). Potassium is the major mineral with an average of about one third of the total (Anklam, 1998; Bogdanov et al., 2007; Conti et al., 2007; Olaitan et al, 2007; Silva et al., 2009), followed by calcium, sodium, phosphorus, magnesium, iron, manganese and copper (Bogdanov et al., 2008; Olaitan et al., 2007). Trace elements like aluminium, iodine, chloride, fluorine, bromine, barium, among others, are also present in honey (Alvarez-Suarez et al., 2010; Bogdanov, 2011). The mineral composition depends on the environment, geographic location and botanical species (Alvarez-Suarez et al., 2010; Anklam, 1998; Bogdanov et al., 2007; González-Miret et al., 2005). In fact, honeys from light blossom commonly have lower mineral content than dark honeys such as honeydew, chestnut and heather (Bogdanov et al., 2007).

Organic Acids

Organic acids comprise gluconic acid, resulting from the oxidation of glucose by glucose oxidase (Bogdanov, 2011; Olaitan et al., 2007), followed in minor concentrations by pyruvic, malic, citric, succinic and fumaric acids (Bogdanov, 2011). These acids account for 0.5 % of the dry matter (Bogdanov et al., 2008; Olaitan et al., 2007), for the acidity of honey and its characteristic taste (Anklam, 1998).

Honey acidity is also dependent on the botanic species (Küçük et al., 2007) and time of harvest (de Rodríguez et al., 2004). The presence of osmophilic yeasts adapted to high osmotic pressures, such as high sugar concentrations, may be responsible for the increase in acidity (de Rodríguez et al., 2004). So, low acidity, below the maximum limit of 50 mmol/kg, indicates absence of undesirable fermentations (Finola et al., 2007). Most honeys are acidic, with pH ranging from 3.4 to 6.1, and an average value of 3.9 (Bogdanov, 2011; Iurlina and Fritz, 2005). However, this parameter is not directly related to the free acidity due to the

buffer capacity of honey (de Rodríguez et al., 2004) which is dependent on phosphates, carbonates and other minerals of honey.

Nitrogen compounds

Amino acids, peptides, proteins and nucleic acids derivatives are the major nitrogenous substances in honey. Amino acids content corresponds to about 10 g/kg (Alvarez-Suarez et al., 2010). Amino acids composition of honey is highly variable depending on its origin, thus amino acid profile is a good indicative of the botanical and geographical origin of honey (Alvarez-Suarez et al., 2010; Anklam, 1998; Chua et al., 2015). Proline is the major amino acid in honey, corresponding to values between 50 % and 85 % of total free amino acids (Alvarez-Suarez et al., 2010; Anklam, 1998). Proline content should be above 200 mg/kg; values below 180 mg/kg indicate potential adulteration of honey by sugar addition (Bogdanov, 2011). Besides proline, 26 other amino acids have been identified in honey: glutamic acid, aspartic acid, glutamine, histidine, glycine, arginine, tryptophan, cysteine, among others (Alvarez-Suarez et al., 2010; Anklam, 1998). The protein content is relatively low, approximately 2 g/kg to 4 g/kg (Bogdanov 2011). Proteins in honey are mainly enzymes: invertase, diastase, glucose oxidase, catalase (Anklam, 1998), α-glucosidase, β-glucosidase (Won et al., 2008). Some enzymes come from the bees during the process of honey ripening (Bogdanov, 2011). The enzymes, diastase and invertase are important for assessing honey quality, since are used as indicatives of honey freshness. Diastase catalyses the hydrolysis of starch into disaccharides and monosaccharides and it is relatively stable to heat and storage, and invertase catalyses the hydrolysis of sucrose to glucose and fructose. The hydrogen peroxide, H_2O_2, antibacterial factor found in honey, is regulated by the enzymes glucose oxidase and catalase. Thus, the enzymatic activity may indicate exposure to heat during processing and storage of honey (Bogdanov, 2011).

Vitamins

The vitamin content in honey is low and varies with the floral origin (Ciulu et al., 2011). Most are water-soluble vitamins due to aqueous nature of honey and a low percentage of lipids (León-Ruiz et al., 2013). Vitamins C (ascorbic acid), B1 (thiamine) and B2 (riboflavin), B6 (pyridoxine), B3 (niacin), B5 (pantothenic acid), K (phyllochinon) have been reported in honey (Alvarez-Suarez et al., 2010; Bogdanov et al., 2008; León-Ruiz et al., 2013; Olaitan et

al., 2007). Ascorbic acid in the main vitamin found in honey with concentrations ranging from 22 mg/kg to 25 mg/kg (Bogdanov et al., 2008) and it is found in almost all honeys. On the other hand, Ciulu et al. (2011) observed a marked association between the concentration of vitamins B3 and B5 and the botanical origin of the product.

Phenolic compounds

Honey contains a diversity of phenolic compounds as secondary constituents, such as flavonoids, phenolic acids and phenolic acid derivatives. The main polyphenols are the flavonoids, in concentrations that can vary between 0.6 g/kg and 4.6 g/kg, and are mainly found in honey produced in dry and high temperature conditions (Bogdanov et al., 2008). The flavonoids present in honey are essentially flavanones and flavones, namely myricetin, tricetin, quercetin, hesperetin, lutein, kaempferol, pinocembrine, chrysin, pinobanksin, genkvanin, galangin, apigenin, naringenin (Anklam, 1998; Arráez-Román et al., 2006; Baltrušaitytė et al., 2007; Bertoncelj et al., 2007; Bogdanov et al., 2008; Estevinho et al., 2008; Yao et al., 2004). The phenolic acids are found in concentrations ranging from 0.01 mg/kg to 10 mg/kg (Anklam, 1998). The predominant phenolic acids are gallic and *p*-coumaric, being caffeic, ferulic, chlorogenic, ellagic, syringic, vanillic, *p*-hydroxybenzoic and cinnamic acids minor constituents (Baltrušaitytė et al., 2007;. Bertoncelj et al., 2007; Estevinho et al., 2008; Tomás-Barberán et al., 2001).

The composition in flavonoids of some honeys, such as heather, citrus, or chestnut, can be used for determining its botanical origin (Escriche et al., 2011; Tomás-Barberán et al., 2001). Dark coloured honeys contain more phenolic acids derivatives but less flavonoids than light coloured ones (Bogdanov, 2011). In fact, heather honeys are characterized by high concentrations of benzoic, phenylacetic, mandelic and β- phenyllactic acids (Anklam, 1998). Considerable differences in composition and concentration of phenolic among unifloral honeys have also been found (Bogdanov, 2011). For instance, hesperetin proved to be a useful marker for the floral origin of citrus honey; kaempferol a marker for rosemary honey, abscisic acid for heather honey and homogentisic acid for strawberry-tree (*Arbutus unedo*) honey (Tomás-Barberán et al., 2001).

The phenolic content of honey is highly related with its bioactive properties, namely antioxidant and antimicrobial activities. The antioxidant activity of honey has been reported by numerous authors (Al et al., 2009; Al-Mamary et al., 2002; Alzahrani et al., 2012;

Baltrušaitytė et al., 2007; Bertoncelj et al., 2007; Estevinho et al., 2008; Gorjanović et al., 2013; Kishore at al., 2011; Küçük et al., 2007; Meda et al., 2005; Ruiz-Navajas et al., 2011). Others, provided evidence of antibacterial activity of honey against pathogenic bacteria resistant to antibiotics (Basualdo et al., 2007; Kumar et al., 2005; Lusby et al., 2005; Moussa et al., 2012; Mulu et al., 2004; Nzeako and Hamdi, 2000; Sherlock et al., 2010; Taormina et al., 2001; Voidarou et al., 2011) and against food spoilage bacteria (Mundo et al., 2004).

Volatile compounds

Volatile compounds of honey are derived from the botanical specie or nectar source, from the transformation process carried out by bees, from heating or handling during processing and storage or from microbial and environmental contamination (Bogdanov, 2011; Escriche et al., 2012; Manyi-Loh et al., 2011).

Aroma compounds are present at very low concentrations, mainly as complex mixtures of volatile components with different functionality and relatively low molecular weight (Cuevas-Glory et al., 2007). Indeed, more than 300 volatile compounds have been identified in different honeys, including hydrocarbons, aldehydes, alcohols, ketones, acids, esters, benzenes derivatives, furans and pyrans, norisoprenoids, terpenes and sulphur compounds (Cacho et al., 2015; Castro-Vázquez et al., 2009; Manyi-Loh et al., 2011).

Usually, monofloral honeys possess highly individual aroma profiles when compared to multifloral ones (Kaškonienė and Venskutonis, 2010). The volatile profile represents a chemical fingerprint of monofloral honey since the nature and amount of volatile compounds are related to the floral source (Bianchi et al., 2011; Escriche et al., 2011; Jerkovic et al., 2009). So, the determination of volatile compounds has been used to differentiate honeys according to botanical origin (Aliferis et al. 2010; Bianchi et al., 2011; Castro-Vázquez et al., 2009; Escriche et al., 2012; Jerković et al., 2009) and geographical origin (Aliferis et al., 2010; Karabagias et al., 2014; Stanimirova et al., 2010). The differences between the geographic sources could be attributed to climatic conditions and to the surrounding flora; nevertheless, the volatile compounds seem to contribute more to the differentiation of honey according to botanical origin, than country of origin (Juan-Borrás et al., 2014). In fact, a considerable number of volatiles have been referred as possible markers of the following monofloral honeys: acacia, chestnut, eucalyptus, heather, lime and sunflower (Radovic et al., 2001); strawberry-tree (Bianchi et al., 2005); thyme (Odeh et al., 2007); citrus, eucalyptus and

lavender (Castro-Vázquez et al., 2009; Escriche et al., 2011). For example, carvacrol and α-terpinene seem to be important in tilia honey, α-pinene and 3-methyl-2-butanol in sunflower and cis-linalool oxide in acacia honey (Juan-Borrás et al., 2014).

Colour

The determination of honey colour is a useful classification criterion for unifloral honeys, since it is related with the contents of phenolic and flavonoids and minerals (Alvarez-Suarez et al., 2010; Baltrušaitytė et al., 2007; Bertoncelj et al., 2007; Gomes et al., 2010). The mineral content influences the colour and the taste; honeys with higher quantity of minerals have darker colour and stronger taste (González-Miret et al., 2005). The colour of honey, that depends on the processing used, temperature and/or time of storage (Olaitan et al., 2007), ranges from white-water, extra-white, white, extra clear amber, light amber, amber to dark amber (Bertoncelj et al., 2007). However, it is important to ascertain that colour's intensity increases during storage due to Maillard reactions, caramelization of fructose and reactions with polyphenolic compounds (Bertoncelj et al., 2007; Shafiee et al., 2013).

The natural microbiota of honey

The microbial population of honey includes microorganisms that come from the environment, soil, plants, and pollen, and those that usually colonize the digestive tract of bees (primary sources of contamination) (Kačániová et al., 2009; Olaitan et al., 2007; Snowdon and Cliver, 1996). Thus, microbial population of honey includes fungi (yeasts and moulds) and spore-forming bacteria (Kačániová et al., 2009; Snowdon and Cliver, 1996). The intestine of bees contains high numbers of Gram-positive bacteria (*Bacillus, Bacteridium, Streptococcus* and *Clostridium* spp.) and Gram-negative bacteria (*Achromobacter, Citrobacter, Enterobacter, Erwinia, Escherichia coli, Flavobacterium, Klebsiella, Proteus,* and *Pseudomonas)* and lower number of yeasts (Al-Waili et al., 2012). Additionally, microbial contamination may also have origin in secondary sources, such as human handling, containers and equipment, insects, animals and water (Kačániová et al., 2009; Snowdon and Cliver, 1996). Possible routes of microbial contamination include air (during packaging), handlers (from skin infections and faecal contamination), cross-contamination (from animals or animal products) and equipment (including residues of food and water) (Snowdon and

Cliver, 1996). While primary sources of contamination are very difficult to control, the secondary sources can be controlled with proper hygiene and good manufacturing practices.

The survival of microorganisms is influenced by honeys' chemical composition, particularly by the low water content. Indeed, this parameter hampers microbial growth, especially of bacteria, which are generally less tolerant to high osmotic pressure, when compared to fungi (Olaitan et al., 2007). Also, the low pH and the high sugar content play a key role in the survival and growth of microorganisms (Al-Waili et al., 2012; Bogdanov, 2011; Iurlina and Fritz, 2005).

Even though bacteria can survive in this natural product, they are unlikely to replicate (Snowdon and Cliver, 1996). As consequence, the detection of high numbers of vegetative bacteria might be indicative of recent contamination by a secondary source (Iurlina and Fritz, 2005). The consumption of honey contaminated with *C. botulinum* spores is especially dangerous for infants and children, with many reported cases of infant botulism. Although honey itself does not contain the toxin, the spores can theoretically build the toxin after digestion in infants until one year old (Bogdanov, 2011).

Moulds, or filamentous fungi, normally associated with honey include the genera *Penicillium*, *Aspergillus*, *Cladosporidium* and *Mucor* (Kačániová et al., 2009; Popa et al., 2009). These microorganisms can survive but do not tend to grow in honey (Snowdon and Cliver 1996). The species *Bettsya alvei*, *Ascosphaera apis* and *Ascosphaera major* may indicate recent contamination by inadequate bee hive management practices (Finola et al., 2007)

Honey naturally contains different osmotolerant/osmophilic yeasts that grow at low pH values and are not inhibited by high osmotic pressure. Most of yeasts isolated from this environment include species of the genera *Saccharomyces*, *Debaromyces*, *Hansenula*, *Lipomyces*, *Pichia*, *Schizosaccharomyces*, *Torula* and *Zygosaccharomyces* (Snowdon and Cliver, 1996). Although studies on the quantification of yeast in honey are scarce, the values reported are normally low. In fact, it were found less than 10 colony forming units (*CFU*) of yeasts per gram, in honeys of central Argentina (Finola et al., 2007) and an average of 12/g in crude honey from India (Pota and Aruna, 2013) while in honey from Brazil the number of yeasts varied from $CFU = 5\times10^2$/g (Sereia et al., 2010) to $CFU = 1.5\times10^5$/g (Rall et al., 2003).

Osmophilic or osmotolerant yeasts have the ability to convert honeys' glucose and fructose into ethanol, carbon dioxide and acids, making the product unsuitable for consumption. According to the literature, honey should follow the quality criteria: maximum

yeast counting of 5×10^4/g and maximum glycerol content of 300 mg/kg; ethanol concentration should be less than 150 mg/kg (Bogdanov, 2011). Honey with moisture content less than 17.1 % is safe of fermentation risk regardless yeast count, however a value above 20 % means that the honey is always in danger of fermentation occurrence (Bogdanov, 2011).

Mead production

The production of mead involves several steps that are presented in the diagram of Figure 1.1.

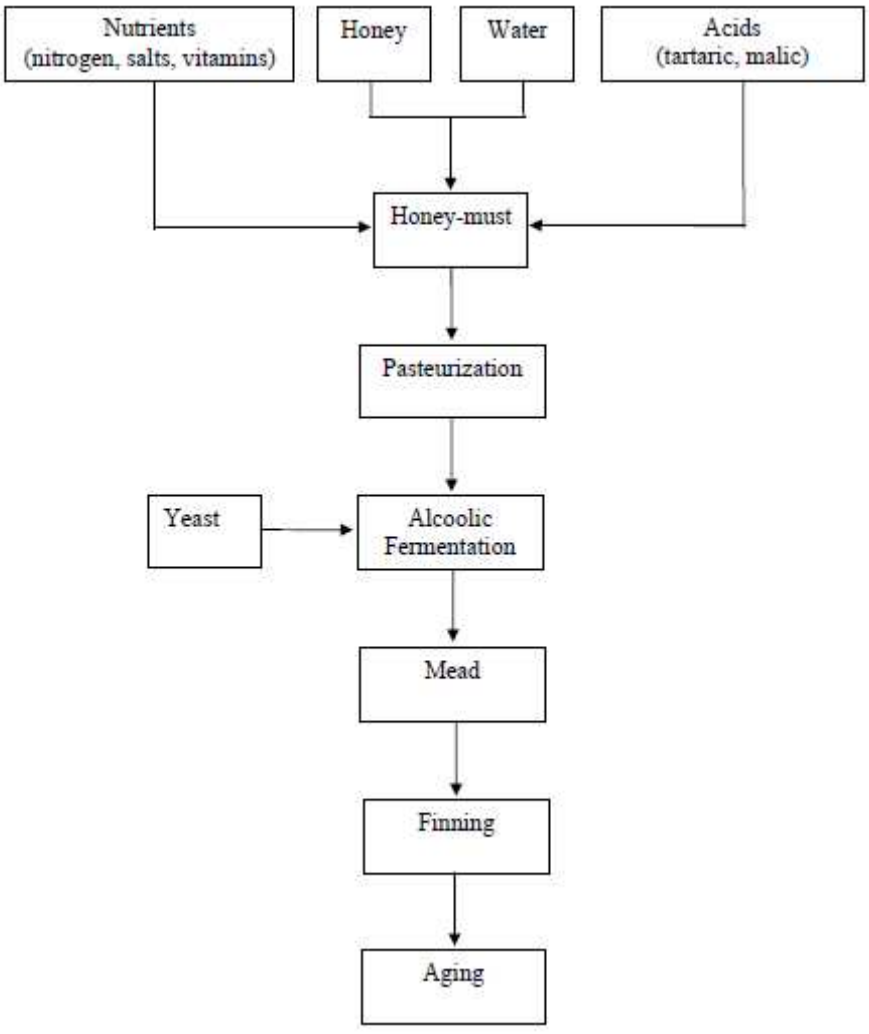

Figure 1.1. Diagram of mead production.

Initially, honey is diluted with water in a proportion that depends on the type of mead desired. In most processes, honey-must starts with *ºBrix* between 20 and 23 (Chen et al., 2013; Mendes-Ferreira et al., 2010; Morales et al., 2013; Pereira et al., 2013, 2014a, 2104b and 2015; Qureshi and Tamhane, 1986; Roldán et al., 2011; Wintersteen et al., 2005). Spices or herbs, either as an extract or directly can be added prior or during the process (National Honey Board, 2001).

After that dilution, a mixture of nutrients, nitrogen, minerals and growth factors may be added if necessary, in order to stimulate yeast growth and fermentation. Also the adjustment of acidity may be done to obtain a better balance between sweetness and acidity. In general, the acids used for honey-must adjustment are: citric acid (Sroka and Tuszyński, 2007), malic acid (Pereira et al., 2013, 2014a, 2104b and 2015) or tartaric acid (Pereira et al., 2009; Roldán et al., 2011). A mixture of tartaric and malic acids may be used not only to adjust the acidity but also to increase the buffer capacity of honey-must (Mendes-Ferreira et al., 2010). The must is subsequently sanitized, being pasteurization one of the most commonly used methods (Mendes-Ferreira et al., 2010; Pereira et al., 2013, 2014a, 2104b and 2015; Wintersteen et al., 2005). In alternative, other techniques are used with the aim of controlling or inactivating most wild microorganisms, including the addition of potassium metabisulphite (Roldán et al., 2011) and sulphur dioxide (Gomes et al., 2013; Pereira et al., 2009; Ukpabi, 2006), or the boiling of must (Navrátil et al., 2001; Sroka and Tuszyński, 2007; Ukpabi, 2006). After honey-must treatment, it is inoculated with selected strains of *Saccharomyces cerevisiae*, from culture collections or active dry yeasts available in the market. Fermentations are conducted at temperatures ranging from 22 ^{o}C to 25 ^{o}C and are daily monitored to reduce the risk of premature fermentation arrest. The duration of fermentation depends on the type of honey, the nutrients added to honey-must, the amount of inoculum size and the fermentation conditions. After completion of alcoholic fermentation, mead must be clarified by centrifugation or by using fining agents such as bentonite, isinglass, egg white, gelatine, and casein, and filtered before bottling. Fining agents are applied to obtain limpid and clear mead, eliminating substances in suspense as well as protein instability (Castillo-Sánchez et al., 2008). Aging is important in mead production, particularly for the development of favourable aroma compounds, generally moving from a harsh, acidic, unpleasant taste to a smooth, to a mellow beverage with a nice bouquet and fragrance (National Honey Board, 2001). The length of aging can go from months to years, depending on the type of mead. In general, lighter meads

will be ready sooner while darker, sweet meads and those with higher alcohol content will need more time to fully develop (National Honey Board, 2001).

Control of honey-must fermentation

S. cerevisiae metabolizes glucose and fructose through the Embden-Meyerhoff pathway with the formation of 2 moles of pyruvate per mole of hexose. Then pyruvate is decarboxylated by pyruvate decarboxylase to acetaldehyde, which is reduced to ethanol with the concomitant oxidation of NADH coenzyme formed in the oxidation of glyceraldehyde-3-phosphate to 1,3-di-phosphogliceric acid. The effective ethanol yield depends on the strain, as well as on the fermentative conditions, among which the temperature and wort composition. In addition to ethanol, *S. cerevisiae* produces small amounts of glycerol, higher alcohols, diacetyl, acetoin, 2,3-butanediol, succinic acid and traces of acetic acid, lactic acid and acetaldehyde (Boulton et al., 1996, Mendes-Ferreira et al., 2011; Phaff et al., 1978) which have strong impact in final taste and aroma composition.

Despite the excellent properties of honey, mead production faces several problems, namely, slow or premature fermentation arrest, lack of uniformity of the final product, and production of yeast off-flavours. Many factors might be related with these problems, such as honey variety, medium composition (vitamin, minerals and nitrogen content), fermentative yeast and fermentation conditions (temperature and pH) (Ramalhosa et al., 2011).

The influence of the honey type was already evaluated in mead production (Pereira et al., 2009). Light honey, comparatively to dark one, has a deficiency in the amount of nitrogen compounds and in the content of minerals that must be fulfilled by supplementation taking into account the yeasts requirements.

Yeasts

The unpredictable nature of spontaneous fermentation and stock fermentation may be associated with the risk of undesirable flavour occurrence (Chen et al., 2013). Hence, inoculation with selected yeasts is a common practice to control the nature and quantity of fermentation products, particularly metabolites that impair the final quality of the product. The yeasts used in mead production are usually strains of *S. cerevisiae* with suitable characteristics as required for wine and beer production: vigorous fermentative activity,

tolerance to ethanol and sulphur dioxide, tolerance to temperature variation and ability to flocculate easily after completion of fermentation. In a previous work, fermentative abilities of five strains of *S. cerevisiae* isolated from Portuguese honey were compared to wine commercial strain (Pereira et al., 2009). According to the results, the performance of the strains isolated from honey was similar to the observed in those available in market, which are used in the production of other beverages. Even though most mead is produced using commercial yeast strains, the traditional mead produced in some African countries still use natural fermentation conducted by microorganisms initially present in the substrates and fermentation equipment. Fortunately, the yeasts *S. cerevisiae* (Teramoto et al., 2005) and *Kluyvermyces bulgaricus* (Bahiru et al., 2006) appear to be the dominant microorganisms in this traditional beverage.

In wine, mixed culture fermentation has been exploited to enhance aroma and flavour and to obtain different types and styles of the product (Fleet, 2003). This practice has been recently tested in mead production by Chen et al. (2013), who successfully used multiple yeast inoculations with different strains.

The use of reduced inoculum of *S. cerevisiae* can be associated with sluggish and stuck fermentations (Carrau et al., 2010). So, in order to provide evidence to this claims, Pereira et al. (2013) studied the effect of the inoculum size on yeast fermentation performance, as well as on mead composition and the volatile compounds production. The increasing of pitching rate resulted in significant fermentation time saving, even though high inoculums could lead to lower production of desirable aromatic compounds.

Nutrient supplementation

The problems of honey-must fermentation are considered to be due to deficiency of nitrogen, minerals and other growth factors (Gupta and Sharma, 2009). The correction of these nutritional deficiencies may reduce stress sensitivity of yeast, improving fermentation performance (Gibson, 2011).

Vitamins, whose concentration is not usually limiting, are required by yeast cells for many enzymatic reactions (Alfenore et al., 2002; Sablayrolles, 2009). Minerals are required as cofactors for several metabolic pathways influencing the rate of sugar conversion (Pereira et al., 2010). Nevertheless, nitrogen deficiency has been reported as the major cause of stuck or sluggish fermentations in grape juice fermentations (Beltran et al., 2005; Mendes-Ferreira et

al., 2011), since nitrogen affects yeast growth, yeast fermentation rate and fermentation length (Bely et al., 1994). Nitrogen concentration also regulates the formation of by-products, such as H_2S, fatty acids, higher alcohols, and esters, among others, which affect the chemical and sensorial proprieties of the alcoholic beverage (Crépin et al., 2012; Mendes-Ferreira et al., 2011; Torrea et al., 2011). In alcoholic fermentation, *S. cerevisiae* normally requires a minimum of 267 mg/L, expressed as nitrogen, for complete fermentation of a must containing 200 g/L of hexoses (glucose + fructose), in an industrially reasonable time (Mendes-Ferreira et al., 2004). In spite of this, there are differences in the nitrogen demand according to the industrial yeast strain, or the quality of the nitrogen source or the must sugar concentration (Manginot et al., 1998; Martínez-Moreno et al., 2012).

Taking into account all the knowledge used in wine production, honey-must was optimised by supplementing it with potassium tartrate, malic acid and diammonium phosphate (DAP) and fermentation time was reduced to 11 days (Mendes-Ferreira et al., 2010). Even under these improved conditions, the available sugars were not completely consumed by yeasts and a certain amount of residual assimilable nitrogen remained in all of the meads, even in controls in which no nitrogen was added, suggesting that other factors could account for the reduced yeast activity in honey-must fermentations. Besides reducing the fermentation length and increasing specific growth rate of yeasts, the addition of DAP to honey-must can contribute to the enhancement of the fruity character of mead (Pereira et al., 2015).

In fact, the supplementation of nitrogen deficiencies with DAP addition is a practice widespread in mead production (Ilha et al., 2000; Morales et al., 2013; Pereira et al., 2013, 2014a and 2014b). In other cases, the honey-must nutritional deficiencies are supplemented in the form of commercial nutrients (Navrátil et al., 2001; Pereira et al., 2009; Wintersteen et al., 2005). In the fermentation of longan mead, the addition of commercial nutrients containing yeast hulls, yeast extract, DAP, vitamin B1, magnesium sulphate, folic acid, niacin and calcium pantothenate Chen et al. (2013) only attained high fermentation rates. Also using commercial nutrients, Gomes et al. (2013) detected high sugar consumption and high production of ethanol, acetic acid, and glycerol with a concentration of 0.88 g/L.

There are references in literature about other natural supplements that can be added to mead to improve yeast growth or yeast fermentative activity: black rice, a natural nutrient for yeast, as a source of fungal glucoamylase (Koguchi et al., 2009; Teramoto et al., 2005); fruit juices as source of acids and growth factors (Gupta and Sharma, 2009) or even pollen (Roldán

et al., 2011). In this study, the addition of pollen improved fermentation rates, ethanol yield, and final sensory attributes.

Yeasts immobilization in mead production

Whole-cell immobilization may be defined as the physical confinement or localization of intact cells to certain defined region of space with the preservation of some desired catalytic activity (Kourkoutas et al., 2004). Microorganism's immobilisation methods have gained attention in the last few decades and are being successfully applied in the alcoholic beverage production. The use of these techniques has made it possible to reduce labour requirements, to simplify time-consuming procedures, and thereby to reduce costs (Diviès and Cachon, 2005).

To be attractive for industrial purpose the methodology must be: robust, not susceptible to contamination, able to impart correct flavour changes to the beverage, not liable to cause oxidation of the product and use commercially acceptable supports and organisms (Diviès and Cachon, 2005).

To obtain the desired product it is fundamental to select a suitable support for cell immobilization, and the choice depends on the process in which it will be applied as well as the process conditions (Genisheva et al., 2014a). Generally, four major categories of immobilization techniques can be distinguished, based on the physical mechanism employed: attachment or adsorption on solid carrier surfaces, entrapment within a porous matrix, self-aggregation by flocculation (natural) or with cross linking agents (artificially induced), and cell containment behind barriers (Genisheva et al., 2014a; Pilkington et al., 1998).

In comparison with free cells, the immobilization may induce alterations in cell growth, physiology and metabolic activity, may affect their tolerance to stress factors and the formation of aroma compounds. Mass transfer limitations by diffusion, disturbances in the growth pattern, surface tension and osmotic pressure effects, reduced water activity, cell-to-cell communication, changes in the cell morphology, altered membrane permeability are some factors considered responsible for alterations through immobilization (Kourkoutas et al., 2004).

Immobilized yeast cells have not been widely used in mead production. Indeed, only a few studies have been reported on this theme. Up to our knowledge, the pioneer work on this matter was conducted by Qureshi and Tamhane (1985) using whole cells of *S. cerevisiae*

immobilized in calcium alginate gels to produce mead. The optimum pH for alcohol production was 4.5 or 5.5 when used free or immobilized cells, respectively. The authors produced mead for a period of more than 3 months using immobilized cells, thus reducing the problems of contamination and secondary fermentation associated with traditional mead production. Later, the same authors (Qureshi and Tamhane, 1986) used two series reactors separately packed with immobilized cells of *S. cerevisiae* and *Hansenula anomala* to produce meads of controlled quality, and reduced the time period of production and eliminated the costlier aging process.

Navrátil et al. (2001) used a two-column packed-bed system with an entrapped ethanol-tolerant distillery yeast of *S. cerevisiae* to provide higher ethanol productivity and thus to make the process more efficient. Yeast cells were immobilised in calcium pectate, which has higher mechanical stability than calcium alginate. The system enabled to increase fermentation rate and allowed to produce mead in a continuous mode.

The capacity of two sodium alginate concentrations, 2 % and 4 %, to immobilize *S. cerevisiae* yeast strains QA23 and ICV D47, in the context of mead production was investigated by Pereira et al. (2014a). Neither of the alginate concentrations was able to prevent cell leakage from the beads. Even so, at the end of the fermentation, the number of cells entrapped in the beads was higher than the number of free cells, and the total 4 % alginate bead wet weight was significantly higher than the 2 % alginate bead wet weight. The fermentation length was 120 h for both yeast strains and the evaluation of mead quality showed that the yeast strain had significantly more influence on the physicochemical characteristics than the alginate concentration.

To avoid cell leakage, it was assessed the potential of application of immobilised yeast cells on single-layer Ca-alginate or double-layer alginate-chitosan for mead production (Pereira et al., 2014b). Minor differences were detected in the fermentation length and in the rate between fermentations conducted with free or immobilised cells, even though higher concentrations of viable cells were achieved in immobilised systems. The double-layer alginate-chitosan had no advantage compared with the single-layer Ca-alginate, as the number of free cells in the medium, resulting from cell leakage, was similar. Meads obtained with entrapped yeast cells presented less ethanol and glycerol and more acetic acid, presenting larger amounts of volatile compounds. Immobilised cells produced meads with higher concentrations of fruity characteristics compounds such as ethyl octanoate and ethyl

hexanoate; however the concentrations of undesirable compounds, namely ethyl acetate, octanoic and hexanoic acids, in such meads were also higher.

Aroma of Mead

The aroma profile is one of the most typical features of a food product, both for its organoleptic quality and authenticity (Alvarez-Suarez et al., 2010). The aroma of mead has contributions from honey, inoculated yeast and technological processes (Chen et al., 2013; Gupta and Sharma, 2009; Pereira et al., 2013, 2014b and 2015).

Honey-derived volatiles

The honey quality, that is crucial in the consumers' assessment, is strongly dependent on the botanical and geographical origin of the product (Bogdanov et al., 2008; Manyi-Loh et al., 2011). Honey aroma is very complex and involves several volatile compounds, however not all have a significant impact on the aroma. In general, the impact of a given compound depends on the extent to which the concentration exceeds its odour threshold. It is important to state that some synergistic and/or antagonistic interactions between different components may occur, and thus, even compounds present in low concentrations may contribute to honey aroma (Kaškonienė and Venskutonis, 2010; Manyi-Loh et al., 2011) In order to determine the influence of the volatile compounds on overall honey aroma, odour activity values (*OAV*) should be assessed by dividing the concentration of each compound by its perception threshold. Only the compounds with *OAV*s greater than 1 (or near) may have contributed to honey aroma (Manyi-Loh et al., 2011). The same volatile compounds identified in various honey samples can be characterized by a wide range of aroma descriptors, for example, from bitter, rancid, or fishy, to sweet and flowery (Table 1.1).

Table 1.1. Odour descriptors of some volatile compounds found in honeys (*sources*: Kaškonienė and Venskutonis, 2010; Manyi-Loh et al., 2011).

Volatile compound	Odour descriptor
Benzaldehyde	Bitter almond; fragrant; aromatic; sweet; marzipan
Benzeneacetaldehyde	Harsh; green
γ-butyrolactone	Woody; toasty; caramel
Carvacrol	Pungent; warm
p-Cymenene	Citrus; pine
β-damascenone	Fruity; sweet; honey
Decanal	Strong; sweet; orange peel odour; citrus taste; soap; fat
Dimethyl disulphide	Vegetable; cabbage; putrid
Dimethyl sulphide	Cabbage; sulphuric; gasoline; sweet; honey; acrid; cooked vegetables
Dimethyl trisulphide	Powerful; fish; diffusive
Ethyl acetate	Ethereal; sharp; wine-brandy-like; reminiscent of pineapple
Ethyl butyrate	Sweet; fruity; pineapple
Furfural	Bread; almond; sweet; woody; fragrant; fruity; cherry
Heptanoic acid	Rancid; sour; sweet-like; fatty
Hexanol	Balsamic; aromatic herb
Hotrienol	Hyacinth; balsamic; aromatic herb
Isophorone and ketoisophorone	Spicy
Lilac aldehyde	Flowery; fresh
Linalool	Sweet; floral; lavender; refreshing; citrus; orange; forest; geranium
2-methylbutanal	Sweet; musty; aldehydic
3-methylbutanal	Sweet; musty; aldehydic
Nonanal	Citrus; fatty; floral; green
Nonanol	Green; sweet; oily
Oak lactone	Woody; toasty; caramel
Octanal	Fat; soap; lemon; green
Pantolactone	Woody; toasty; caramel
Phenylacetaldehyde	Sweet; honey-like
2- phenylethyl acetate	Flowery; sweet; champagne
Sinensal	Sweet; orange
Spathulenol	Cheese; hay

Sensory evaluation, based mainly in attributes of aroma and taste, is one of the most useful tools in honey characterization (Anupama et al., 2003; Castro-Vázquez et al., 2009;

Castro-Vázquez et al., 2010). Some of the aroma attributes proposed have been floral, fruity, candy, waxy, resin, wood, citric, acidic, spicy, balsamic, caramel, herbaceous, coffee/chocolate, cheese, chemical and fermented, among others. The attributes sweet, acid, astringent, ripe fruit, toasty caramel, woody and spicy have been selected for taste characterisation. Honeys from different geographical and botanic origins differ regarding their sensory profile. For instance, the attributes flowery, fruity, waxy, jaggery-like, chemical and caramel notes were the major variables among honey samples from India (Anupama et al., 2003). Castro-Vázquez et al. (2009) identified the volatile compounds and the sensory descriptors that are more representative of different monofloral honeys namely, citrus, rosemary, eucalyptus, lavender, thyme and heather. These authors verified that citrus honeys were characterised by higher amounts of linalool derivatives and by fresh fruit and citric aromas; eucalyptus honeys had hydroxyketones and *p*-cymene derivatives together with cheese and hay aromas; lavender honeys had mainly hexanal, nerolidol oxide and coumarin and the sensorial attributes balsamic and aromatic herbs aromas; finally, heather honeys were characterised by high contents of benzene and phenolic compounds and ripe fruit and spicy aromas. Regarding chestnut honeys from Spain it was verified that the volatile composition and sensory profile are greatly influenced by the geographic origin, i.e., honeys from the Spanish north-east presented significantly higher concentrations of aldehydes, alcohols, lactones and volatile phenols which are associated with herbaceous, woody, and spicy notes; honeys from the north-west area showed superior levels of terpenes, esters and some benzene derivatives, closely related with honey-like, floral and fruity notes (Castro-Vázquez et al., 2010).

Fermentation yeast-derived volatiles

During the alcoholic fermentation, yeasts produce a range of compounds with strong sensorial importance in the quality of the final product. Fermentative compounds, resulting from the metabolic activity of yeasts, represent quantitatively, the majority of volatile compounds in wines (Vilanova and Oliveira, 2012); therefore these microorganisms play an important role in the development of wine aroma. In the last decade, some research has been conducted on volatile compounds formation during mead fermentation. The production of volatile compounds is affected by several factors including the yeast strain (Chen et al., 2013; Teramoto et al., 2005), cell condition (free or immobilised) (Pereira et al., 2014b) and

inoculum size (Pereira et al., 2013), as well as by the fermentation conditions (Wintersteen et al., 2005). In addition, the type of honey (Šmogrovičová et al., 2012; Vidrih and Hribar, 2007; Wintersteen et al., 2005), and the honey-must composition/formulation (Sroka and Tuszyński, 2007; Mendes-Ferreira et al., 2010; Roldán et al., 2011; Pereira et al., 2015) can also modulate the formation of volatile compounds. The volatile compounds produced by yeasts are: alcohols, organic acids, esters, volatile fatty acids, carbonyl compounds, volatile phenols, among others.

Alcohols

Alcohols are secondary yeast metabolites and, from a quantitative point of view, are the most important group of volatile compounds produced by yeast during alcoholic fermentation of sugars (Swiegers et al., 2005; Ugliano and Henschke, 2009), inclusive in mead production (Mendes-Ferreira et al., 2010; Pereira et al., 2013, 2014b and 2015; Roldán et al., 2011). Alcohols include 2-methyl-1-propanol (isobutanol), 2-methyl-1-butanol, 3-methyl-1-butanol (isoamyl alcohol) and 2-phenylethanol (with pleasant rose-like aroma), among others (Swiegers et al., 2005; Ugliano and Henschke, 2009). The most predominant alcohol in some meads has been 3-methyl-1-butanol, in concentration ranging from 90 mg/L to 350 mg/L (Chen et al., 2013; Mendes-Ferreira et al., 2010; Pereira et al., 2013, 2014b and 2015; Roldán et al., 2011; Teramoto et al., 2005; Wintersteen et al., 2005), above the odour threshold of 30 mg/L (Guth, 1997; Moreno et al., 2005). Comparatively, lower concentrations of 3-methyl-1-butanol were found in Slovak and South African meads (Šmogrovičová et al., 2012). Other secondary predominant alcohols present in mead are 2-methyl-1-butanol, 2-methyl-1-propanol, 1-propanol and 2-phenylethanol (Mendes-Ferreira et al., 2010; Pereira et al., 2013, 2014b and 2015; Roldán et al., 2011; Teramoto et al., 2005; Vidrih and Hribar, 2007; Wintersteen et al., 2005).

Generally, concentrations of alcohols in mead are below 300 mg/L. Excessive concentrations, above 400 mg/L, may have negative impacts on the aroma and flavour resulting in a strong, pungent smell and taste Swiegers et al., 2005). Mendes-Ferreira et al. (2010) verified an inverse correlation between higher alcohols and nitrogen levels in mead and Roldán et al. (2011) found an increase in alcohols with pollen addition to honey must. Moreover, the immobilisation of yeast cells of *S. cerevisiae* seems to enhance the production of 1-propanol (Pereira et al., 2014b).

Esters

Esters are derived from a reaction between organic or volatile fatty acids and ethanol (ethyl esters) or between acetic acid and higher alcohols (acetates), being largely responsible for wine and fermented beverages fruitiness, and therefore they play an important role in the sensory composition of the related young products (Mendes-Ferreira et al., 2010; Pereira et al., 2013; Ugliano and Henschke, 2009). Ethyl acetate is quantitatively the most important ester found in mead produced in Portugal (Mendes-Ferreira et al., 2010; Pereira et al., 2013, 2014b and 2015), Slovak and South Africa (Šmogrovičová et al., 2012), Spain (Roldán et al., 2011), Slovenia (Vidrih and Hribar, 2007) and Southwest Ethiopia (Teramoto et al., 2005). Ethyl acetate is an ester compound with a solvent-like like odour (Bartowsky and Pretorius, 2009; Meilgaard, 1975) and an odour threshold of 12.3 mg/L (Escudero et al., 2004). Other esters found in mead in minor amounts are isoamyl acetate, 2-phenylethyl acetate, ethyl butyrate, ethyl hexanoate and ethyl octanoate. These esters have pear-drops aromas (isoamyl acetate), honey, fruity, flowery aromas (2-phenylethyl acetate) and fruity, sweet aromas (ethyl butyrate, ethyl hexanoate and ethyl octanoate) (Bartowsky and Pretorius, 2009). However, highly variability in the concentration of these compounds is observed between mead, probably because different commercial yeast strains can produce variable amounts (Swiegers et al., 2005).

Esters production by yeasts increases with nitrogen concentration (Mendes-Ferreira et al., 2010), with the addition of nutrients to honey-must, like pollen (Roldán et al., 2011), and in mead fermented with yeast cells immobilised in single-layer of alginate or double-layer of alginate-chitosan (Pereira et al., 2014b).

Volatile fatty acids

Volatile fatty acids includes a mixture of straight chain fatty acids, resulting from β-oxidation of fatty acids, usually referred to as short chain (C2–C4), medium chain (C6–C10), long chain (C12–C18), and a group of branched-chain fatty acids, from the metabolism of the aminoacids (Ugliano and Henschke, 2009). Acetic acid is quantitatively and sensory the most important volatile fatty acid produced during alcoholic fermentation, accounting for more than 90 % of the total volatile acidity (Bartowsky and Pretorius, 2009). Acetic acid at elevated concentrations imparts a vinegar-like character and it becomes objectionable at concentrations of 0.7 g/L to 1.1 g/L, being the optimal concentration between 0.2 g/L and 0.7 g/L (Swiegers et al., 2005). In mead, it have been reported concentrations of acetic acid less than 0.6 g/L

(Pereira et al., 2009 and 2014b; Sroka and Tuszyński, 2007), although Švecová et al. (2015) found concentrations above 1 g/L in Czech meads. Volatile acidity increases during fermentation mainly as a result of acetic acid synthesis. So, values ranging between 0.4 g/L and 4 g/L have been referred in mead (Mendes-Ferreira et al., 2010; Pereira et al., 2013, 2014b and 2015; Roldán et al., 2011; Šmogrovičová et al., 2012; Wintersteen et al., 2005). As the fatty chain length increases, the volatility decreases and the odour changes from sour to rancid and cheese (Ugliano and Henschke, 2009), characteristic of the compounds hexanoic, octanoic, and decanoic acids. Generally, octanoic acid is the main fatty acid in mead, followed by hexanoic and decanoic acids (Mendes-Ferreira et al., 2010; Pereira et al., 2013 and 2014b; Roldán et al., 2011). This fatty acid has an odour threshold of 0.5 mg/L (Ferreira et al., 2000) and its amount in mead can vary from 0.1 mg/L (Roldán et al., 2011) to 6 mg/L (Mendes-Ferreira et al., 2010). On the other hand, Sroka and Tuszyński (2007), verified that decanoic acid was in higher amounts in mead than octanoic and dodecanoic acids, but all of them in concentration below 30 mg/L. The concentration of medium chain fatty acids was higher in meads supplemented with nitrogen compared to non-supplemented fermentations (Mendes-Ferreira et al., 2010; Pereira et al., 2015; Roldán et al., 2011).

Carbonyl compounds

Yeasts produce various carbonyl compounds from sugar metabolism, being acetaldehyde quantitatively the most important, constituting more than 90 % of the total aldehydes in wines and other alcoholic fermented beverages (Nykänen, 1986). It contributes with ‘bruised apple’ and ‘nutty’ characters, when present at sensory detectable concentrations, but can also be a sign of wine oxidation (Swiegers et al., 2005; Ugliano and Henschke, 2009). This compound has been found in meads produced by *S. cerevisiae* in concentrations between 5 mg/L and 30 mg/L (Pereira et al., 2013, 2014b and 2015; Roldán et al., 2011), always above its perception threshold of 0.5 mg/L (Guth, 1997). However, considerably higher concentrations of this compound were obtained for mead produced by any other yeast specie, *Saccharomyces bayanus*, either in lime mead (608 mg/L) or chestnut mead (1370 mg/L) (Vidrih and Hribar, 2007). In addition, the concentration of this compound appears to be related with the must composition, increasing with the addition of pollen (Roldán et al., 2011) and nitrogen (Pereira et al., 2015).

Volatile phenols

Volatile phenols have a relatively low detection threshold and are, therefore, easily detected due to their pharmaceutical odour (Swiegers et al., 2005). Although they can contribute positively to the aroma of some wines, are better known as off-flavours such "Band-aid", "barnyard" or "stable" (Bartowsky and Pretorius, 2009). The most important volatile phenols are the ethylphenols, 4-ethylguaiacol and 4-ethylphenol, and the vinylphenols, 4-vinylguaiacol and 4-vinylphenol. Higher concentrations of 4-vinylphenol than 4-vinylguaiacol have been found in mead produced with a multifloral dark Portuguese honey, but in concentrations below their detection thresholds (Pereira et al., 2013, 2014b and 2015). This production was not affected by the yeast strain. Also in buckwheat and soy mead was detected 4-methylphenol, but again, in concentrations below the odour threshold (Wintersteen et al., 2005).

Because beverages can contain a very complex set of volatile compounds, to estimate the contribution of an individual compound in the overall aroma it is important the determination of *OAV* (Czerni et al., 2008) The volatile compounds with more influence on mead aroma profile are the alcohols (3-methyl-1-butanol and 2-phenylethanol), esters (mainly ethyl acetate, isoamyl acetate, ethyl butyrate, ethyl hexanoate and ethyl octanoate), medium chain fatty acids (hexanoic, octanoic and decanoic acids) and acetaldehyde (Pereira et al., 2013, 2014b and 2015; Wintersteen et al., 2005). Even though few studies are available about the *OAV*s in mead, the results show that they depend on the inoculum size, yeast cell immobilisation, nitrogen addition to fermentation, as well as on the yeast strain and honey used in mead production.

Sensory evaluation of mead

Aroma volatile compounds play a key role in determining the quality of beverages because are the primary contributors to aroma and produce an effect on sensory characteristics (Andreu-Sevilla et al., 2013; Vilanova et al., 2010). Two main types of methodologies are used for evaluation of quality of food and beverages. The identification and quantification of aroma compounds, as an objective analysis technique, or subjective methods based on human assessment of the quality characteristics of the food (Smyth and Cozzolino, 2013). Sensory

analysis is indispensable for the assessment of food flavour characteristics to identify the significant sensory and quality contributors to food quality and consumer preference (Schmidtke et al., 2010). Overall, the more important sensory characteristics of beverages are the smell, the taste and to a lesser extent, the colour (Robinson et al., 2011) and are performed by a panel of experts or consumers. However, the sensory perception is variable within individuals, the context of the consumer experience and the chemical composition of the product (Schmidtke et al., 2010).

Even though the identification and quantification of aroma compounds in mead produced under different conditions has been assessed (Chen et al., 2013; Mendes-Ferreira et al., 2010; Pereira et al., 2013 and 2014b; Šmogrovičová et al., 2012; Sroka and Tuszyński, 2007; Vidrih and Hribar, 2007; Wintersteen et al., 2005), there is a lack of evidence regarding the sensory quality of the mead produced.

Koguchi et al. (2009) produced mead with honey and black rice and performed the sensory test of the beverages, revealing that mead made from Chinese milk vetch honey was acceptable, while the produced using buckwheat honey was not very palatable. Sensory characteristics of mead produced with cassava (*Manihot esculenta*) floral honey under farm conditions in Nigeria was also assessed (Ukpabi, 2006). In this latter study, the expert test panel included nine food scientists, who commented freely on fresh and stored mead samples. The colour and taste of the meads were generally acceptable and the characteristic after-taste bitterness of samples was both pointed as positive and negative attribute.

Vidrih and Hribar, (2007) studied the sensory properties of three different types of mead produced from chestnut, lime and honeydew varieties of honey. The trained panellists chose the chestnut honey solution as the best raw material for mead production, followed by lime and honeydew honey solutions. After fermentation, honey was added to meads and panellists preferred meads with 80 g/L sugar over meads with 40 g/L reducing sugar (dry mead with no reducing sugar is rather flat in taste and poor in body) and gave the best scores to chestnut and lime types mead. In the chestnut mead the reducing sugars masked the bitterness taste of the raw honey and the fermentation process improved the bouquet of lime mead.

In short, the results of the works on mead sensory analysis indicate that high sugar content is an important requisite to mead' consumers.

The establishment of correlations between instrumental measurements of specific attributes and sensory characteristics may lead to a better understanding of the relationship between volatile composition and sensory properties, which is important to assess the quality

of the beverage (Schmidtke et al., 2010). In this context, Roldán et al. (2011) evaluated the influence of pollen addition on the sensory characteristics of mead, namely on visual (turbidity and colour), aroma (quality and intensity) and taste (quality and intensity) characters and verified that the aroma quality appeared to be related to the volatile compounds. The aroma of control mead was described as floral (associated with 2-phenylethanol) and vinegar-like acid (presence of 3-methylbutyric and hexanoic acids, ethyl acetate and high total and volatile acidity) masking other aromas, which decreased the aroma quality. Mead with high amount of pollen added was characterised by toasted, bitter almond and honey scents that masked all other aromas, principally consistent with its high phenylacetaldehyde levels. Briefly, the pollen addition led to an increase in the volatile contents of meads, consequently improving its sensory profile.

Context, Objectives and Outline

In Portugal beekeeping is a dynamic activity that is in frank expansion in recent years. Even so, it is necessary to continue to valorize the national honey and simultaneously find alternatives for the honey that can not be commercialized. Thus, the mead production may emerge as a great alternative for profit and to add value to honey not suitable for trading. Although mead has been produced since ancient times, its production is empirical and homemade by the beekeepers. The producers found several problems during fermentation, such as, lack of uniformity in the final product, slow or premature fermentations arrest, and the production of "off-flavours". Due to these problems and to the lack of scientific progress in this area, we considered essential optimizing the processes of mead production.

In recent years our team has been focused on the optimization of mead production and some achievements have been attained: yeast strains residents in honey have been selected, their fermentation performance under ethanol, sulphur dioxide and osmotic stress was evaluated; a honey-must formulation has been designed and some problems related with delay or fermentation arrest have been identified and, in a certain way, have been overcame. Taking into account all these factors, the evaluation/ identification of limiting factors of fermentation and the development of new production processes, assume extreme importance in the optimization of mead production. These constitute the main objectives of this work.

To this end, two active dry wine yeasts *Saccharomyces cerevisiae* strains, QA23 and ICV D47, were selected for all the studies performed for this thesis. In the same way it was selected a honey-must formulation from a previous study of our research group (Mendes-Ferreira et al., 2010). Although it was the formulation that provided the best fermentation results, some problems were reported, such as the residual nitrogen and reducing sugar concentrations in the final of fermentation. In order to prove that nothing was limiting in the composition of the medium formulated, it was evaluated the effect of honey-must supplementation with salts and/or vitamins required for yeast growth (**Chapter 2**). The supplementation with salts and/or vitamins had no positive effects on the fermentation and growth profiles or in mead final composition. On the basis of the results obtained, we proceeded to evaluate the impact of a high initial cell density on yeast fermentation performance and mead quality, using five different sizes of inoculum (**Chapter 3**). Our results demonstrate that increasing inoculum size results in significant time savings in the fermentation process, from 24 to 96 h depending on the inoculum size. However, the final

aroma composition was dependent on the yeast strain and inoculum size; an exaggerated inoculum led to lower production of desirable aromatic compounds. Therefore, we continued the studies using yeast cell immobilization given the advantages over free cells reported in literature, such as, increased substrate uptake and protection against inhibitory substances, among others. First, we started the experiments with calcium alginate gels since they are the most widely used matrices for cell entrapment. For that purpose 2 and 4 % of sodium alginate concentrations was tested. The results showed that the alginate concentrations tested have not prevent the phenomenon of cell leakage but the entrapment agent did not cause negative effects on mead production (**Chapter 4**). Thus, further experiments were conducted with yeast cell immobilization in single layer of Ca-alginate (4%) or double layers of alginate-chitosan were tested for mead production (**Chapter 5**). The fermentation profile, cell viability, composition and aroma profile were evaluated in mead fermented with free or immobilized cells. Results showed that the most aromatic meads were the ones produced by immobilized cells, but the undesirable compounds were also higher in these fermentations. To analyse the sensory properties of mead and to relate the volatile compounds identified with differences detected in aroma attributes of mead, several experiments were conducted using high fermentation volumes. The results of volatile compounds formation and the acceptance/preference of taster panel are presented in **Chapter 6**. Finally, in **Chapter 7** are presented the final conclusions, and perspectives.

Altogether, the results of this study are expected to contribute to the optimization of mead production, particularly in solving some problems associated with problematic fermentations. Moreover, it also provides new information that can be very useful for this beverage industry.

CHAPTER 2

Improvement of mead fermentation by honey-must supplementation

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Abstract

Through honey's fermentation, diverse beverages can be obtained, among which 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 i) salts, ii) vitamins or iii) salts + vitamins. The effects of the honey-must formulation on fermentation kinetics, growth profile and physicochemical characteristics of final meads were evaluated. The results showed minor differences in the fermentation profile and length 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 SO_2 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 on the characteristics of the final products.

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 the agricultural practices (Al-Mamary et al., 2002; Anklam, 1998; Arráez-Román et al., 2006; Azeredo et al., 2003; Baltrušaityté et al., 2007; Küçük et al., 2007). Honey contains about 200 different substances, with carbohydrates being the main constituents and minor components are minerals, proteins, vitamins, lipids, organic acids and amino acids (Al-Mamary et al., 2002; Arráez-Román et al., 2006; Küçük et al., 2007; Finola et al., 2007).

The increasing appreciation of beehive products by the consumers has boosted honey production, promoting the economic development of the beekeeping industry (Ramalhosa et al., 2011). 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 the consumers and to increase beekeepers' profits (Küçük et al., 2007).

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 (Iglesias et al., 2014).

Mead's fermentation is a time-consuming process, taking from weeks to months to complete, and the quality of the final product highly variable (Iglesias et al., 2014; Navrátil et al., 2001). 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 (Pereira et al., 2009).

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 (Attfield, 1997; Bauer and Pretorius, 2000; Bisson, 1999).

In mead production, little evidence is available concerning the importance of the supplementation of honey-must with nutrients (Pereira et al., 2009), DAP (Mendes-Ferreira et al., 2010; Pereira et al., 2015) or bee pollen (Roldán et al., 2011) for improving the fermentation rates and the final characteristics of the beverage. Moreover, Pereira et al. (2009) 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 had 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 stress sensitivity of yeast, improving the fermentation performance (Gibson, 2011). 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 (Alfenore et al., 2002).

Owing to this, 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.

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 38ºC according to the manufacturer's instructions and inoculated onto Yeast Peptone Dextrose agar (YPD - 20 g/L glucose, 10 g/L peptone, 5 g/L, yeast extract and 20 g/L agar). Incubation was carried out at 25ºC 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 (Pires et al., 2009) 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, 18th September), the characteristics and satisfactory quality of the honey were assured through an analysis of the following parameters: moisture content, diastase index and hydroxymethylfurfural (HMF) content according to Gomes et al. (2010); pH, acidity and reducing sugars (fructose and glucose) as described by Bogdanov et al. (1997); and electric conductivity and ash content as described by Gomes et al. (2010).

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 (Mendes-Ferreira et al., 2010). 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, 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 in 4 parts to perform the following fermentations:

i) control
ii) control + salts (14 g/L of dipotassium phosphate, 1.23 g/L of magnesium sulphate and 0.44 g/L of calcium chloride)
iii) control + vitamins (100 mg/L of inositol, 2 mg/L of pyridoxine, 2 mg/L of nicotinic acid, 1 mg/L of calcium pantothenate, 0.5 mg/L of thiamine, 0.2 mg/L of riboflavin and 0.125 mg/L of biotin)
iv) control + salts + vitamins

The parameters ºBrix (Optic Ivymen 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 (Organisation Internationale de la Vigne et du Vin, 2006). Yeast assimilable nitrogen (YAN) was determined by the formaldehyde method as previously described (Aerny, 1996). The honey-musts were pasteurised at 65ºC 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 min^{-1}. 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 (Mendes-Ferreira et al., 2010) that consisted of 250 mL flasks filled to 2/3 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 25ºC 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 (Mendes-Ferreira et al., 2009). Fermentations were monitored daily by weight loss as an estimate of CO_2 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 (YPD - 20 g/L glucose, 10 g/L peptone, 5 g/L, yeast extract and 20 g/L agar) plates after incubation at 25ºC 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 deionised water, dried for 24 h at 100°C and stored in a desiccator before weighing. The oenological parameters such as total sulphur dioxide (SO_2), pH, titratable acidity, volatile acidity and ethanol content were determined according to standard methods (Organisation Internationale de la Vigne et du Vin, 2006), and YAN was determined by the formaldehyde method (Aerny, 1996). Determinations of reducing sugars were performed using the 3,5-dinitrosalicylic acid (DNS) 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, were 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 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 (Mendes-Ferreira et al., 2010) 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 Figure 2.1.

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 *Saccharomyces 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.

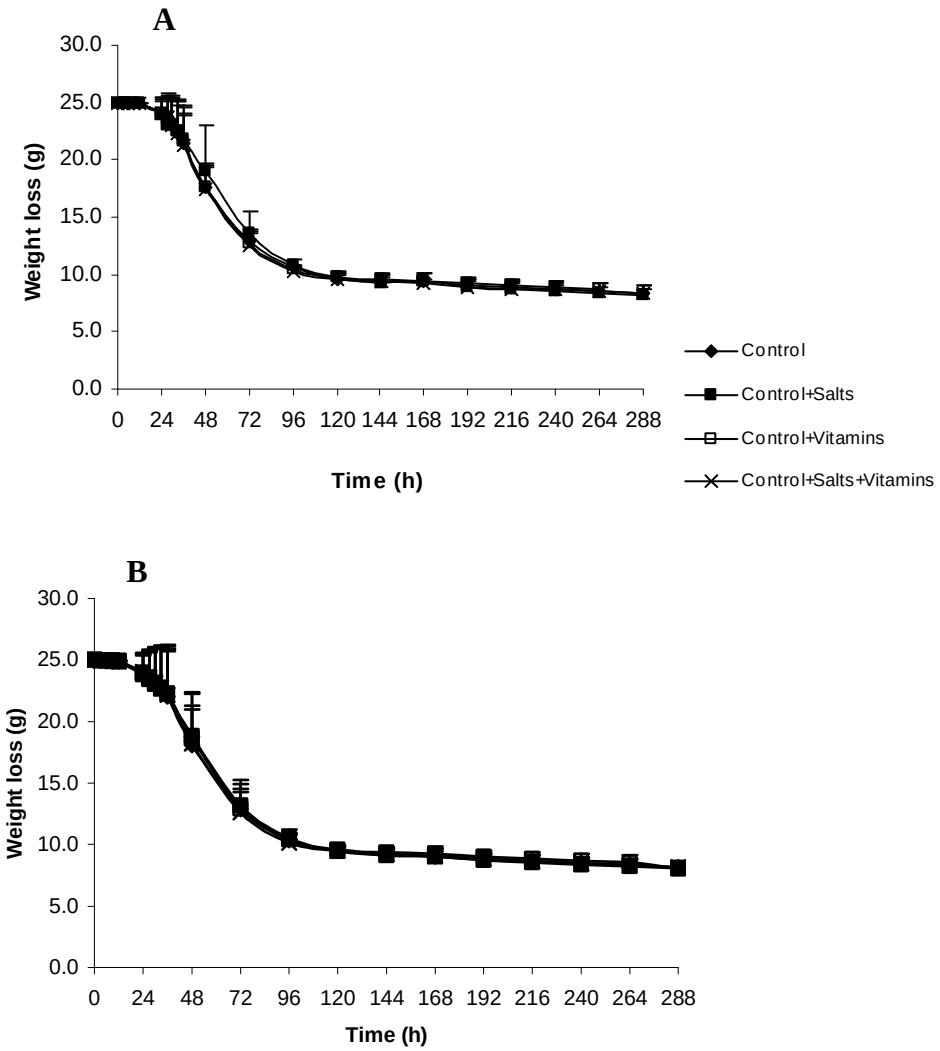

Figure 2.1. Fermentation profiles of *S. cerevisiae* QA23 (A) and ICV D47 (B) in control fermentation and fermentations with honey-must supplemented with salts, vitamins or salts + vitamins.

The effect of supplementation of honey-must with minerals and/or vitamins on the growth of yeasts QA23 and ICV D47 is displayed on Figure 2.2.

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 10^8 CFUs/mL. The growth profile of strain ICV D47 (Figure 2.2B) 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 - 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 (Figure 2.1), 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.

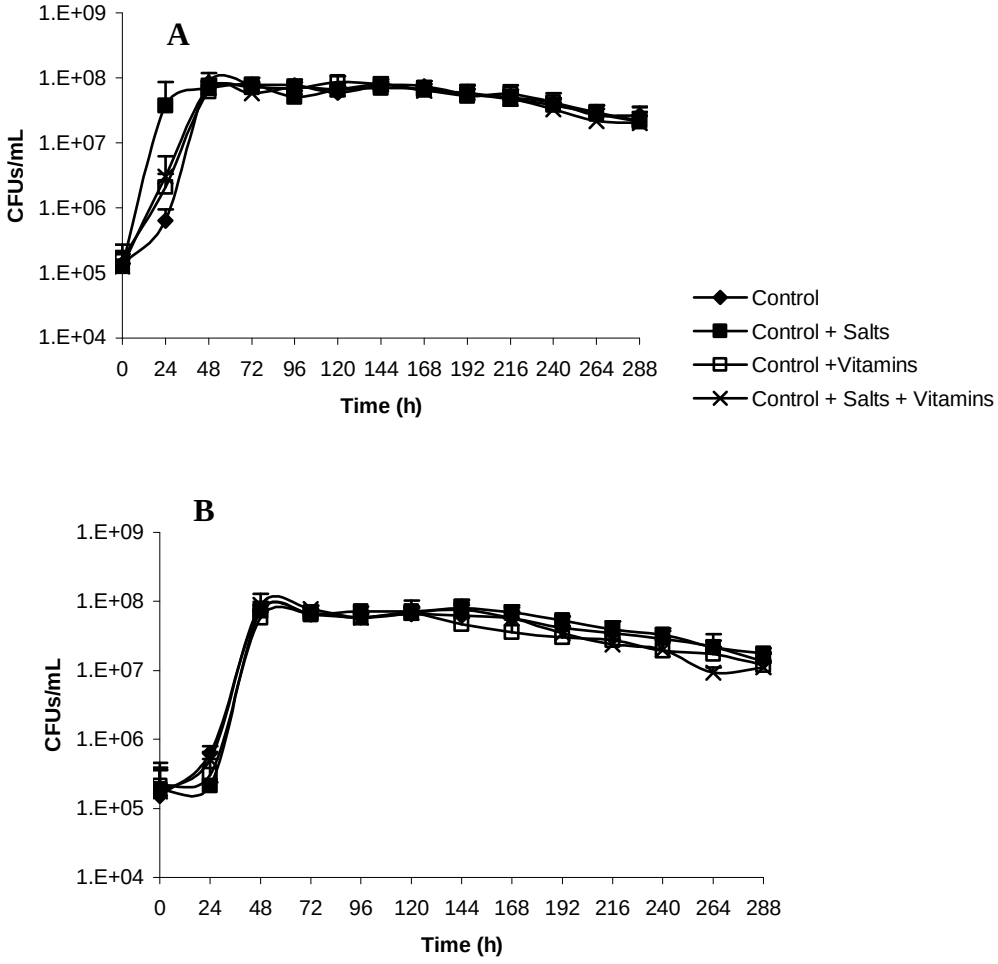

Figure 2.2. Growth profiles of *S. cerevisiae* QA23 (A) and ICV D47 (B) in control fermentation and fermentations with honey-must supplemented with salts, vitamins or salts + vitamins.

Specific nutrients, such as nitrogen, minerals or vitamins, are required to obtain rapid fermentation and high ethanol levels (Alfenore et al., 2002). The minerals, magnesium, calcium and zinc, influence the rate of sugar conversion and are required as cofactors for several metabolic pathways (Pereira et al., 2010). Also, deficiencies in vitamins, especially thiamine and biotin, have been identified as being potentially responsible for fermentation problems, such as slow yeast growth (Alfenore et al., 2002; Maisonnave et al., 2013). However, the supplementation of honey-must with vitamins or salts did not contribute significantly to enhance 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 (Alvarez-Suarez, et al., 2010; Silva et al., 2009), and dark honeys have a higher mineral content (0.2%) than light honeys (0.04%) (Anklam, 1998; Fernández-Torres et al., 2005). In heather honeys (*Erica* sp.) potassium, calcium and phosphorus are the minerals present in the highest levels, with potassium quantitatively being the most important mineral; and can account for 76% of the total mineral content (Fernández-Torres et al., 2005; Silva et al., 2009). The vitamin content in honey is generally low, and includes phyllochinon (K), thiamine (B1), riboflavin (B2), pyridoxine (B6), niacin, panthothenic acid and ascorbic acid (Alvarez-Suarez, et al., 2010; Bogdanov et al., 2008). 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 SO_2 and ethanol, for strains QA23 and ICV D47 are presented in Table 2.1.

Table 2.1. Physicochemical characteristics of honey-must and meads produced by *S. 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
Honey-musts				
pH	3.67 ± 0.06	3.77 ± 0.06	3.67 ± 0.04	3.78 ± 0.04
º Brix (%)	23.20 ± 0.26	23.40 ± 0.26	23.30 ± 0.20	23.23 ± 0.12
Titratable acidity $_{\text{tartaric acid}}$ (g/L)	4.28 ± 0.24	4.13 ± 0.33	4.19 ± 0.26	4.03 ± 0.38
Initial nitrogen $_{\text{YAN}}$ (mg/L)	263.67 ± 4.04	268.33 ± 5.35	269.50 ± 7.00	268.33 ± 11.25
Meads produced by strain QA23				
pH	3.61 ± 0.13	3.64 ± 0.13	3.58 ± 0.12	3.64 ± 0.11
Volatile acidity $_{\text{acetic acid}}$ (g/L)	0.63 ± 0.11	0.67 ± 0.08	0.53 ± 0.21	0.60 ± 0.00
Titratable acidity $_{\text{tartaric acid}}$ (g/L)	7.53 ± 0.15	7.57 ± 0.16	7.09 ± 0.16	7.29 ± 0.35
Final nitrogen $_{\text{YAN}}$ (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
Meads produced by strain ICV D47				
pH	3.55 ± 0.13	3.66 ± 0.11	3.62 ± 0.12	3.68 ± 0.14
Volatile acidity $_{\text{acetic acid}}$ (g/L)	0.57 ± 0.03	0.55 ± 0.05	0.60 ± 0.06	0.56 ± 0.02
Titratable acidity $_{\text{tartaric acid}}$ (g/L)	7.06 ± 0.52	6.69 ± 0.30	6.69 ± 0.40	6.74 ± 0.41
Final nitrogen $_{\text{YAN}}$ (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 significant difference within a line, $p < 0.05$. Lack of a superscript indicates no significant difference, $p > 0.05$.

The low pH and the poor buffer capacity of honey could lead to the decrease of pH during the fermentation (Ramalhosa et al., 2011). The drop of 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 (McConnell and Schramm, 1995). Although the pH was slightly higher in the honey-musts supplemented with salts, probably due 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 (Nikolaou et al., 2006). 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 (Mendes-Ferreira et al., 2010; Pereira et al., 2009; Pereira et al., 2013; Pereira et al., 2014; Roldán et al., 2011; Sroka and Tuszyński, 2007). 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 titrable 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 titrable acidity, in order of 2-3 g/L, during the fermentation of mead has previously been reported (Mendes-Ferreira et al., 2010; Roldán et al., 2011; Sroka and Tuszyński, 2007). The increase in acidity is caused mainly by the synthesis of acetic and succinic acids by yeasts (Sroka and Tuszyński, 2007). The amounts of 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 titrable acidity. As expected, based on acetic acid concentration, the titrable 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 titrable 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 (McConnell and Schramm, 1995; Mendes-Ferreira et al., 2010). Independent of the strain or the honey-must formulation, at the end of all fermentations a concentration of residual nitrogen, between 30 to 40 mg/L, remained in all meads. Mendes-Ferreira et al. (2010) 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 (Anklam, 1998).

Concerning the concentration of SO_2, the strains showed different behaviour in its production during the fermentations. Although no SO_2 was added to the honey-must, its concentration was detected in all of the meads at the end of fermentations. Yeasts can produce less than 10 mg/L, of SO_2 during fermentation, but in certain cases production can exceed 30

mg/L (Ribéreau-Gayon, 2000). For strain ICV D47, no significant differences were observed between the fermentations, with the amount of SO_2 ranging from 14.5 to 15.8 mg/L in the final meads. However, for strain QA23, the concentration of SO_2 in meads supplemented with salts and salts + vitamins was significantly higher. The production of SO_2 can be affected by fermentation conditions such as the nutritional composition of the medium (Eglinton and Henschke, 1996) and the choice of yeast strains (Taylor et al., 1986).

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 (Pereira et al., 2013; Pereira et al., 2015).

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 meads' 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.

CHAPTER 3

High-cell-density fermentation of *Saccharomyces cerevisiae* for the optimisation of mead production

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Abstract

Mead is a traditional drink that contains 8 % to 18 % (v/v) of ethanol, resulting from the alcoholic fermentation of diluted honey by yeasts. Mead fermentation is a time-consuming process and the quality of the final product is highly variable. Therefore, the present investigation had two main objectives: first, to determine the adequate inoculum size of two commercial winemaking strains of *Saccharomyces cerevisiae* for the optimisation of mead fermentation; and second, to determine if an increase in yeast pitching rates in batch fermentations altered the resulting aroma profiles. Minor differences were detected in the growth kinetics between the two strains at the lowest pitching rate. With increasing pitching rates net growth of the strain ICV D47 progressively decreased, whereas for the QA23 the increasing inoculum size had no influence on its net growth. The time required to reach the same stage of fermentation ranged from 24 to 96 hours depending on the inoculum size. The final aroma composition was dependent on the yeast strain and the inoculum size. Fourteen of the twenty-seven volatile compounds quantified could contribute to mead aroma and flavour because their concentrations rose above their respective thresholds. The formation of these compounds was particularly pronounced at low pitching rates, except in mead fermented by strain ICV D47, at 10^6 CFUs/mL. The esters isoamyl acetate, ethyl octanoate and ethyl hexanoate were the major powerful odourants found in the meads. The results obtained in this study demonstrate that yeast strain and inoculum size can favourably impact mead's flavour and aroma profiles.

Keywords: aroma profile, fermentation profile, mead, pitching rate, *Saccharomyces cerevisiae*, yeast growth.

Introduction

Mead is a traditional drink, containing 8 % to 18 % (v/v) of ethanol resulting from the alcoholic fermentation of diluted honey by yeasts. Honey production is an activity of significant economic importance in several regions of Portugal. New honey-based products such as mead must be developed to maintain apiculture as a viable industry. However, when mead is homemade, problems such as a lack of uniformity of the final products arise, probably due to the variability of honey composition between years, refermentation by yeasts or by acetic acid- and lactic acid-producing bacteria, which may increase volatile acidity and abnormal ester production and thus affect the organoleptic qualities of the final product (O`Connor-Cox and Ingledew, 1991).

Mead fermentation is a time-consuming process that often takes several months to complete, depending on the type of honey, yeast strain and honey-must composition (Navrátil et al., 2001). An important objective of mead makers is to reduce the fermentation time without decreasing the quality of their end products. Some studies of mead production optimisation have been performed. Pereira et al. (2009) achieved fermentations within roughly 8 days using dark and light honeys enriched with two different supplements. More recently, Mendes-Ferreira et al. (2010) optimised honey-must preparation for mead production by supplementing the honey-must with potassium tartrate, malic acid and diammonium phosphate (DAP) and were able to reduce the fermentation time to 11 days. Even under these improved conditions, the available sugars were not completely consumed by yeasts and a certain amount of residual assimilable nitrogen remained in all of the meads, even in controls in which no nitrogen was added. In addition, the density of yeast in colony forming units (CFUs) even under the most favourable conditions was never higher than 10^7 CFUs/mL, suggesting that there is something in honey-must that inhibits the growth of yeast.

It has been shown that significant time can be saved in the fermentation process by increasing the pitching rate, *i.e.*, the amount of suspended yeast cells added to a batch fermenter (Verbelen et al., 2009a, b). However, an increase in the pitching rate could also have deleterious side effects on the fermentation performance or on the flavour profile of the final beverage (Verbelen et al., 2009a).

In this study, to further improve the mead fermentation process, the best formulation selected from a previous study (Mendes-Ferreira et al., 2010) was used to investigate the impact of the pitching rate on yeast fermentation performance as well as on the mead

composition and the volatile aromatic compound production. The impact of higher inoculum size was assessed with two active dry wine yeast *Saccharomyces cerevisiae* strains. The strain QA23 was selected because it offers dependability under difficult winemaking conditions and it has low requirements for oxygen and assimilable nitrogen. The strain ICV D47 was used because it has a high fermentation rate, a low production of acetaldehyde and volatile acidity and because it is recommended for mead production. Further details about the strains are given in the website of yeast producer www.lallemand.com.

Material and Methods

Yeast strains

S. 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 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 (Pires et al., 2009) 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, of 18th September), the characteristics and satisfactory quality of the honey were assured through an analysis of the following parameters: moisture content, diastase index and hydroxymethylfurfural (HMF) content according to Gomes et al. (2010); pH, acidity and reducing sugars as described by Bogdanov et al. (1997); and electric conductivity and ash content as described by Sancho et al. (1991).

Preparation of honey-must for fermentation

To obtain an alcoholic beverage with approximately 11 % of ethanol, honey was diluted in natural spring-water obtained in the market (37 % w/v), and mixed to homogeneity as previously described (Mendes-Ferreira et al., 2010). After, any insoluble materials were removed from the mixture by centrifugation (2682.8 $\times g$ 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, USA) and pH was adjusted to 3.7 with malic acid (Merck, Darmstadt, Germany). The nitrogen content was adjusted to 267 mg/L with diammonium phosphate (DAP, BDH Prolabo, Leuven, Belgium). The parameters °Brix, pH, total acidity and assimilable nitrogen concentration were determined, prior and after the adjustments. The honey-musts were pasteurised at 65°C for 10 min and then immediately cooled. No sulphur dioxide was added to the honey-musts.

Inoculum preparation

Starter cultures were prepared by rehydration of 10 g of active dry yeast into 100 mL of honey-must at 38 ºC according to the manufacturer's instructions to obtain 10^8 CFUs/mL.

Fermentation conditions and monitoring

The appropriate amounts of inoculum were pitched into the honey-must to obtain five different pitching rates: (PR1) 1.5×10^5 CFUs/mL, (PR2) 10^6 CFUs/mL, (PR3) 10^7 CFUs/mL, (PR4) 4×10^7 CFUs/mL and (PR5) 10^8 CFUs/mL. All fermentations were carried out in triplicate using a previously described system (Mendes-Ferreira et al., 2010) that consisted of 250 mL flasks filled to 2/3 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 22 °C under permanent but moderate shaking (120 rpm min^{-1}) mimicking real industrial environment. Aseptic sampling for assessing fermentation and growth parameters was performed using a syringe-type system as previously described (Mendes-Ferreira et al., 2009). Fermentations were daily monitored by weight loss as an estimate of CO_2 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 Heλios) and for counting their CFUs in solid Yeast Peptone Dextrose agar (YPD–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. Determinations of reducing sugars were performed using the 3,5-dinitrosalicylic acid (DNS) method with glucose as the standard. 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.

Analyses performed at the end of fermentation

The culture dry weight was determined from triplicate samples of 14 mL centrifuged in pre-weighed tubes at 3890.1×*g* for 10 min, washed twice with sterile deionised water, dried for 24 h at 100 °C and stored in a desiccator before weighing. The maximum fermentation rate was determined from the slope of the linear dependence of the steepest decline in weight at the corresponding time points.

The oenological parameters such as total sulphur dioxide (SO_2), pH, titratable acidity, volatile acidity and ethanol content were determined according to standard methods (Organisation Internationale de la Vigne et du Vin, 2006). Yeast assimilable nitrogen (YAN) was determined by formaldehyde method as described elsewhere (Aerny, 1996). After clarification, 10 mL of sample was transferred into a 50 mL beaker and diluted with 15 mL of water. The pH was adjusted to 8.1 with NaOH 0.1 M and 2.5 mL of formaldehyde with pH 8.1 was added. After 5 min the pH was adjusted again to 8.1 by titration with NaOH 0.05 M. Assimilable nitrogen was calculated using the formula:

$$\text{YAN (mg/L)} = [(\text{vol. NaOH}) \times (\text{conc. NaOH}) \times 4 \times 1000] / (\text{sample volume})$$

Analysis of mead aromatic compounds

Mead produced with five different yeast pitching rates 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 made 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 ionisation detector (FID) and a capillary column CP-Wax 57 CB (50 m × 0.25 mm; 0.2 µm film thickness) was used. The temperature 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 °C to 220 °C at 3 °C min^{-1} and finally 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. (2006). In a 10 mL culture tube (Pyrex, ref. 1636/26MP), 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 mm × 4.8 mm) were added. 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 = 5118.5 min, 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 ionisation 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 °C to 234 °C at a rate of 3 ºC/min, raised from 234 °C to 250 ° C at 10 °C/min and finally maintained at 250 ºC 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.

Statistical analysis

An analysis of variance (ANOVA) with Type III sums of squares was performed using the GLM (General Linear Model procedure) of the SPSS software, version 17.0 (SPSS, Inc.). The fulfilment of the ANOVA requirement of homogeneity of variance was evaluated by means of Levene's test. All dependent variables were analysed using a one-way ANOVA with or without Welch correction, depending on whether the requirement of the homogeneity of variances was fulfilled. The main factor studied was the effect of pitching rate on the physicochemical characteristics and aromatic compounds of meads and if a statistically significant effect was found, the means were compared using Tukey´s honestly significant difference multiple comparison test or Dunnett's T3 test, depending on whether equal variances could be assumed. All statistical tests were performed at a 5 % significance level.

Results and Discussion

Honey-must was diluted in spring water to obtain an alcoholic beverage with approximately 11% (v/v) ethanol (Mendes-Ferreira et al., 2010). On the basis of the results obtained in previous assays, adjustments in assimilable nitrogen and pH were performed to optimise the yeasts' fermentation performance. To evaluate the impact of a high initial cell density on yeast fermentation performance and mead quality, four different pitching rates were used to obtain the following CFUs/mL: 10^6, 10^7, 4×10^7 and 10^8 of *Saccharomyces cerevisiae*. In parallel, a control fermentation was carried out with 1.5×10^5 CFUs/mL for comparison.

Effect of pitching rate on yeast growth

Figure 3.1 contains the growth profiles of both strains QA23 and ICV D47 under the various conditions tested. As expected, the maximum cell biomass and the maximum number of CFUs were obtained at a pitching rate of 10^8 CFUs/mL.

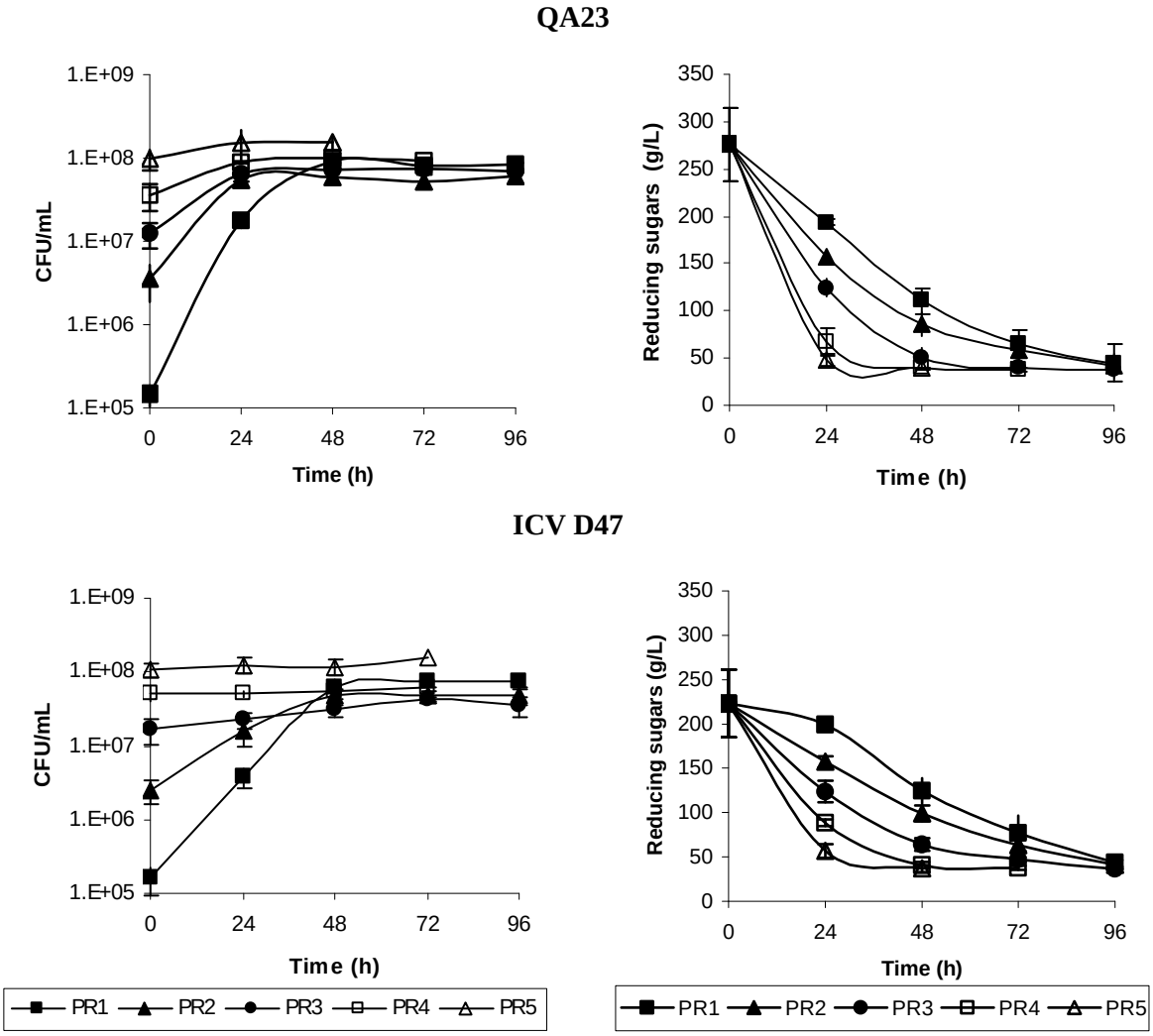

Figure 3.1. Growth and sugar consumption profiles of *S. cerevisiae* QA23 and *S. cerevisiae* ICV D47 in fermentations with different yeast pitching rates. Pitching rates: (PR1) 1.5×10^5 CFUs/mL, (PR2) 10^6 CFUs/mL, (PR3) 10^7 CFUs/mL, (PR4) 4×10^7 CFUs/mL, and (PR5) 10^8 CFUs/mL.

Yeast net growth, calculated by subtracting the initial CFU count from the maximum count, was higher for the lowest pitching rate (1.5×10^5 CFUs/mL) for both strains (Figure 3.2). The net growth of the strain ICV D47 progressively decreased with increasing pitching rates, in agreement with previous studies using high-cell-density fermentations for wine-making (Carrau et al., 2010) or brewing (Verbelen et al., 2009a). At the highest pitching rates (4×10^7 or 10^8 CFUs/mL), no detectable increase in yeast growth was observed, which could be explained by a cell-to-cell contact mechanism at high-cell-density of *S. cerevisiae* (Verbelen et al., 2009a). Contrary, the yeast net growth values were similar for the QA23 strain for the other pitching rates tested, 10^6, 10^7 and 4×10^7 CFUs/mL, suggesting that the increasing

inoculum size had no influence on its net growth. Taken together these results, it seems that each strain responded differently to cell density being ICV D47 more sensitive to space limitation. Further studies are required to establish why one yeast strain is less able to compete for space than another.

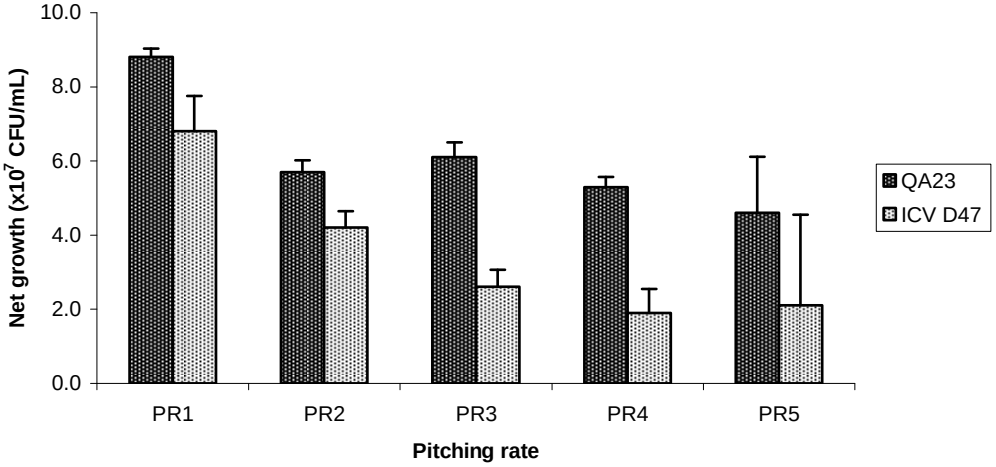

Figure 3.2. Net yeast growth (the maximum CFU count minus the initial inoculum size) of mead fermentations with the yeast strains *S. cerevisiae* QA23 and *S. cerevisiae* ICV D47. Pitching rates: (PR1) 1.5×10^5 CFUs/mL, (PR2) 10^6 CFUs/mL, (PR3) 10^7 CFUs/mL, (PR4) 4×10^7 CFUs/mL, and (PR5) 10^8 CFUs/mL.

Minor differences in growth kinetics were detected between the strains. At the three highest pitching rates (10^7 - 10^8 CFUs/mL), the number of CFUs of the strain ICV D47 remained constant throughout the fermentation; however, a slight increase was observed in the strain QA23 at pitching rates of 10^7 CFUs/mL and 4×10^7 CFUs/mL. Moreover, at all pitching rates tested, the final CFUs of the strain ICV D47 were lower than those attained by the strain QA23, as shown in Figure 3.2. Although specific growth rates were similar in both strains, ICV D47 (0.15 h^{-1}) and QA23 (0.16 h^{-1}), at the lowest pitching rate, fermentation conducted by the former strain started later. Nevertheless, both strains entered into stationary phase 48 h after inoculation and the yeast cells remained viable after 168 h (results not shown). Entrance into stationary phase cannot be determined solely by the nitrogen depletion of the media because at the end of fermentation, some residual assimilable nitrogen remains in the media. Moreover, the amount of residual nitrogen was almost independent of the pitching rate or yeast strain used. This observation has been reported by Mendes-Ferreira et al. (2010) using the same honey-must formulation but a different yeast strain.

To verify whether the phenolic compounds were the inhibitors of yeast growth, the honey-musts were filtered through a SEPAK C-18 cartridge to partially remove phenolic

compounds before inoculation with the same strains and under the same conditions detailed above in the material and methods section. No differences in yeast growth characteristics or fermentative performance were detected, suggesting that probably other compounds present in honey are the interfering agents (results not shown).

Effect of pitching rate on yeast fermentation profiles

Figure 3.1 contains the fermentation kinetics of *S. cerevisiae* QA23 and *S. cerevisiae* ICV D47 after pitching at five different rates. The time required to reach the same stage of fermentation in all pitching rates tested was approximately 96 h for the two smallest inocula, 72 h for the pitching rate of 10^7 CFUs/mL, 48 h for the pitching rate of 4×10^7 CFUs/mL and approximately 24 h for the highest pitching condition. Therefore, a 100-fold increase in the number of cells pitched reduced the fermentation time by 3 days, suggesting that the increase in pitching rate strongly decreased the duration of fermentation. Different results were obtained by Verbelen et al. (2009a), who achieved a 78% reduction of fermentation time by increasing the pitching rate to fourfold of that used in conventional brewery fermentations (20×10^6 viable cells/mL). In fact, in the present work, the fermentation time was reduced by 34% at a fourfold higher yeast concentration. This result is in agreement with previous observations that in addition to exogenous nitrogen, other factors could account for reduced yeast activity in honey-must fermentations (Mendes-Ferreira et al., 2010). Given the difficulty in inocula preparation associated with the problems inherent in mead clarification and the accumulation of products or metabolic by-products to a growth-inhibitory level (Riesenberg and Guthke, 1999), high-cell-density fermentations may be of limited utility.

The fermentation profile of the strain IVC D47 at different pitching rates was largely similar to that of the strain QA23; however, a slight increase in fermentation time was observed (Figure 3.1). For example, at the highest pitching rate of the strain ICV D47, the fermentation lasted for approximately 10 h more than with QA23.

The differences between the two strains were more obvious at the highest pitching rates (Figure 3.2). Hence, young cells of strain ICV D47 were not generated in the high-cell-density fermentations. It is clear that the strain QA23 had fermentation and growth characteristics suitable to mead production, confirming its adaptation to the stressful conditions of wine-making. The strain QA23 consumed sugars more efficiently than did the strain ICV D47, especially at the smallest inoculum size (1.5×10^5 CFUs/mL). In fact, the

strain ICV D47 experienced longer lag phases and lower sugar consumption on the first day of fermentation.

For both strains and for all experimental conditions, although the fermentation had ceased, approximately 30 to 40 g/L of residual sugar remained in the media (Figure 3.1 and Tables 3.1 and 3.2). Residual sugars were determined by GC-MS and the results confirmed the presence of the non-fermentable sugars usually found in honeys (i.e., not glucose, fructose or sucrose, results not shown). In a previous study, very low residual glucose and fructose levels were detected in meads obtained from dark honey enriched with different supplements (Pereira et al., 2009).

Effect of pitching rate on mead composition

At the end of the alcoholic fermentations, samples were analysed to evaluate the meads' final compositions. Tables 3.1 and 3.2 present certain parameters recognised as essential for the composition and stability of meads, such as pH, volatile and titratable acidity, SO_2 concentration and ethanol concentration of the final meads fermented by QA23 and ICV D47, respectively. Both strains behaved similarly with respect to these characteristics, with the exceptions of pH and volatile acidity.

The pH values of the meads obtained with strain QA23 were identical to the honey-must (3.7) and remained constant during all fermentations, indicating that this parameter was not influenced by the pitching rate. On the contrary, meads obtained with strain ICV D47 demonstrated a slight decrease in pH to a range of 3.49 to 3.55.

The volatile acidity of meads fermented with QA23 ranged from 0.25 to 1.38 g/L (Table 3.1) and increased with pitching rate. In contrast, the ICV D47 strain yielded slight variations in the volatile acidity among meads (0.39 - 0.60 g/L), but again, the highest value was detected at the highest pitching rate. The results obtained with ICV D47 are very interesting, considering that volatile acidity should be minimised to avoid vinegar-like off-flavours (Mendes-Ferreira et al., 2010). At the pitching rate of 10^7 CFUs/mL, the volatile acidity of mead fermented by strain QA23 (0.63 g/L) was lower than that obtained by Sroka and Tuszyński (2007) after 7 d of fermentation (0.75 g/L) with other *S. cerevisiae* strain. In fact, the production of acetic acid, which is quantitatively and sensorially the most important volatile fatty acid produced during alcoholic fermentation, is influenced by several factors, including yeast strain and inoculum size (Ugliano and Henschke, 2009).

Table 3.1. Physicochemical characteristics of honey-must and meads obtained after fermentation by *S. cerevisiae* QA23 at different pitching rates. Pitching rates: (PR1) 1.5×10^5 CFUs/mL, (PR2) 10^6 CFUs/mL, (PR3) 10^7 CFUs/mL, (PR4) 4×10^7 CFUs/mL, and (PR5) 10^8 CFUs/mL. Data are the means of triplicate fermentations ± S.D.

Honey-musts	**Prior adjustment**	**After adjustment**
pH	4.54 ± 0.14	3.70 ± 0.01
º Brix	22.87 ± 0.15	23.17 ± 0.35
Titratable acidity $_{\text{tartaric acid}}$ (g/L)	0.70 ± 0.09	4.64 ± 1.07
Initial nitrogen $_{\text{YAN}}$ (mg/L)	49.00 ± 7.00	277.67 ± 14.15

Meads	**PR1**	**PR2**	**PR3**	**PR4**	**PR5**	***P*-value**
pH	3.66 ± 0.07	3.71 ± 0.07	3.70 ± 0.10	3.70 ± 0.13	3.70 ± 0.10	0.964
Volatile acidity $_{\text{acetic acid}}$ (g/L)	0.25 ± 0.02 [a]	0.33 ± 0.03 [a]	0.63 ± 0.04 [b]	1.02 ± 0.12 [c]	1.38 ± 0.16 [d]	<0.001
Titratable acidity $_{\text{tartaric acid}}$ (g/L)	6.74 ± 0.62	6.68 ± 0.83	7.18 ± 1.01	7.48 ± 0.93	7.76 ± 0.88	0.509
Final nitrogen $_{\text{YAN}}$ (mg/L)	29.17 ± 5.35	32.67 ± 2.02	33.25 ± 2.47	37.33 ± 2.02	37.33 ± 2.02	0.049
Total SO_2 (mg/L)	26.45 ± 3.91	24.32 ± 4.62	24.32 ± 5.58	23.04 ± 4.43	24.32 ± 6.77	0.949
Ethanol (% vol)	10.03 ± 0.38	10.33 ± 0.12	10.10 ± 0.14	10.33 ± 0.23	10.13 ± 0.31	0.555
Final reducing sugar (g/L)	37.87 ± 1.30	40.52 ± 0.70	37.41 ± 2.43	37.87 ± 1.56	38.91 ± 1.68	0.214

a–d Means within a line with different superscripts differ, $P < 0.05$.

P-values are those for the effect of pitching rate on physicochemical characteristics of mead, from one-way ANOVA analysis. If there was a significant effect of pitching rate on the analysed parameters, then the means were compared with Tukey's test because equal variances could be assumed ($P > 0.05$ by means of the Levene test).

Table 3.2. Physicochemical characteristics of honey-must and meads obtained after fermentation by *S. cerevisiae* ICV D47 at different pitching rates. Pitching rates: (PR1) 1.5×10^5 CFUs/mL, (PR2) 10^6 CFUs/mL, (PR3) 10^7 CFUs/mL, (PR4) 4×10^7 CFUs/mL, and (PR5) 10^8 CFUs/mL. Data are the means of triplicate fermentations ± S.D.

Honey-musts	**Prior adjustment**	**After adjustment**
pH	4.39 ± 0.21	3.71 ± 0.03
º Brix	22.57 ± 0.25	22.93 ± 0.12
Titratable acidity $_{\text{tartaric acid}}$ (g/L)	0.69 ± 0.06	4.08 ± 1.03
Initial nitrogen $_{\text{YAN}}$ (mg/L)	64.17 ± 29.35	281.17 ± 15.78

Meads	**PR1**	**PR2**	**PR3**	**PR4**	**PR5**	***P*-value**
pH	3.52 ± 0.16	3.54 ± 0.16	3.52 ± 0.19	3.49 ± 0.20	3.55 ± 0.19	0.996 *
Volatile acidity $_{\text{acetic acid}}$ (g/L)	0.39 ± 0.08	0.46 ± 0.12	0.53 ± 0.17	0.49 ± 0.10	0.60 ± 0.03	0.262 *
Titratable acidity $_{\text{tartaric acid}}$ (g/L)	6.35 ± 1.02	6.24 ± 1.13	6.48 ± 1.12	6.58 ± 1.08	6.70 ± 0.93	0.984 *
Final nitrogen $_{\text{YAN}}$ (mg/L)	31.50 ± 9.26	29.17 ± 12.29	36.17 ± 22.77	30.33 ± 14.15	42.00 ± 12.62	0.821 *
Total SO_2 (mg/L)	25.60 ± 2.56	25.60 ± 3.39	25.60 ± 4.62	22.61 ± 2.66	26.45 ± 3.91	0.716 *
Ethanol (% vol)	9.70 ± 0.26	10.10 ± 0.56	10.03 ± 0.38	10.27 ± 0.06	10.37 ± 0.06	0.092 **
Final reducing sugar (g/L)	35.27 ± 2.86	39.08 ± 1.90	37.06 ± 3.16	37.58 ± 2.26	38.68 ± 2.44	0.437 *

Lack of a superscript indicates no significant difference, $P > 0.05$.

* *P*-values are those for the effect of pitching rate on physicochemical characteristics of mead, from one-way ANOVA analysis. If there was a significant effect of pitching rate on the analysed parameters, then the means were compared with Tukey's test because equal variances could be assumed ($P > 0.05$ by means of the Levene test).

** *P*-values are those for the effect of pitching rate on physicochemical characteristics of mead, from one-way Welch ANOVA analysis. If there was a significant effect of pitching rate on the analysed parameters, then the means were compared with the Dunnett T3's test because equal variances could not be assumed ($P < 0.05$ by means of the Levene test).

The titratable acidity, total sulphur dioxide, alcohol content and final reducing sugars were similar in all of the meads fermented by either strain. Despite the increase in titratable acidity, the accentuation of which in meads fermented with QA23 indicates a high production of acids by this strain, no statistically significant differences were detected among the five pitching rates. The amounts of sulphur dioxide produced by the strain QA23 or by the strain ICV D47 were similar in all assays and were independent of the pitching rate. The total absence of SO_2 is rare, even when sulphite is not added prior to fermentation, because yeast produce small quantities of this compound during fermentation that in certain cases can exceed 30 mg/L (Ribéreau-Gayon et al., 2000).

Despite the differences in net growth of QA23, the final ethanol content was nearly identical for all of the assays and varied from 10.03 % (v/v) at the lowest pitching rate (1.5×10^5 CFUs/mL) to 10.33 % at 10^6 and 4×10^7 CFUs/mL (Table 3.1). The yeast strain ICV D47 produced less ethanol but similarly varied from 9.70 % (1.5×10^5 CFUs/mL) to 10.37 % (10^8 CFUs/mL). The discrepancy between the expected and the detected ethanol is explained by the residual non-fermentable sugars, approximately 35 - 40 g/L that remained in meads.

The residual nitrogen in all of the meads produced by both strains varied between 29.17 and 42.0 mg/L and there were no significant differences between the pitching rates tested. These results are in agreement with the concentrations of residual nitrogen detected by Mendes-Ferreira et al. (2010) using the same formulation of honey-must as in this study. Regardless of the inoculum size, the yeast assimilable nitrogen (YAN) consumed (initial nitrogen minus final nitrogen) was identical in all of the assays for both strains (Figure 3.3). The estimated content of residual nitrogen may be the result of the quantification of nitrogen compounds not assimilable by the yeasts and in particular the amino acid proline. In fact, the formaldehyde method used here has a recovery rate of only 23 % for proline (Filipe Ribeiro and Mendes-Faia, 2007); however, this amino acid represents 50 - 85 % of the total nitrogen content of honey (Anklam, 1998).

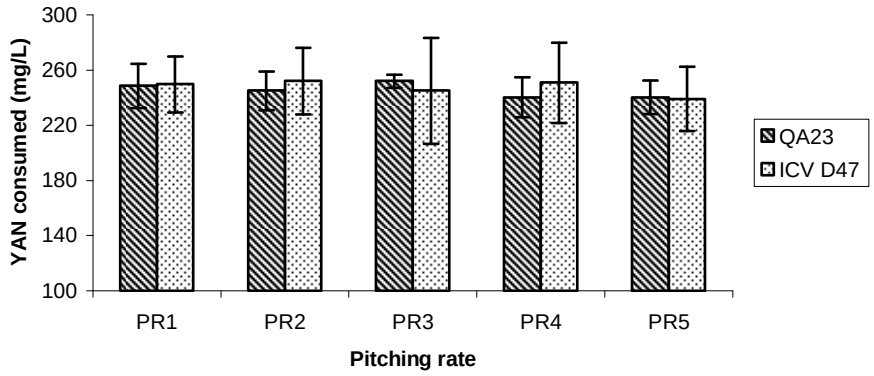

Figure 3.3. Total yeast assimilable nitrogen (YAN) consumption (initial nitrogen minus final nitrogen) of mead fermentations with the yeast strains *S. cerevisiae* QA23 and *S. cerevisiae* ICV D47. Pitching rates: (PR1) 1.5×10^5 CFUs/mL, (PR2) 10^6 CFUs/mL, (PR3) 10^7 CFUs/mL, (PR4) 4×10^7 CFUs/mL, and (PR5) 10^8 CFUs/mL.

The results of our mead composition analysis indicate not only that an increase in pitching rates is not recommended but also that the strain ICV D47 appears to be more suitable for the production of high quality meads, although the strain QA23 showed a better fermentation profile.

Effect of pitching rate on mead aroma profile

Seven major volatile compounds, including acetaldehyde, ethyl acetate, methanol, 1-propanol, 2-methyl-1-propanol, 2-methyl-1-butanol and 3-methyl-1-butanol were analysed by GC-FID. The minor compounds quantified by GC-MS were ethyl butyrate, isoamyl acetate, ethyl hexanoate, ethyl lactate, 3-ethoxy-1-propanol, ethyl octanoate, isobutyric acid, butanoic acid, ethyl decanoate, 3-(methylthio)-1-propanol, ethyl phenylacetate, 2-phenylethyl acetate, ethyl dodecanoate, hexanoic acid, 2-phenylethanol, octanoic acid, 4-vinylguaicol, decanoic acid, 4-vinylphenol and dodecanoic acid.

The effects of the pitching rate and the strain on mead volatile aromatic composition are presented in Table 3.3 for strain QA23 and in Table 3.4 for strain ICV D47. A total of twenty-seven fermentative aroma compounds which contribute to the sensorial qualities of alcoholic beverages, including alcohols, esters, volatile phenols, volatile fatty acids and carbonyl compounds were identified and quantified in these meads.

Table 3.3. Concentration of volatile compounds in meads obtained after fermentation by *S. cerevisiae* QA23 at different pitching rates. Pitching rates: (PR1) 1.5×10^5 CFUs/mL, (PR2) 10^6 CFUs/mL, (PR3) 10^7 CFUs/mL, (PR4) 4×10^7 CFUs/mL and (PR5) 10^8 CFUs/mL. Data are the means of triplicate fermentations ± S.D.

	PR1	PR2	PR3	PR4	PR5	*P*-value
Alcohols (mg/L)						
3-methyl-1-butanol	167.92 ± 5.53 ^{b}	133.64 ± 10.43 ^{a}	128.45 ± 9.64 ^{a}	117.57 ± 11.71 ^{a}	122.90 ± 9.71 ^{a}	0.001 *
2-methyl-1-propanol	22.52 ± 2.33 ^{a}	19.90 ± 2.81 ^{a}	27.99 ± 2.96 ^{a}	41.24 ± 8.14 ^{ab}	62.57 ± 1.26 ^{b}	<0.001 **
2-methyl-1-butanol	21.48 ± 1.02 ^{a}	16.16 ± 0.98 ^{a}	22.56 ± 3.27 ^{ab}	28.33 ± 2.62 ^{bc}	31.75 ± 3.53 ^{c}	<0.001 *
1-propanol	17.95 ± 1.69 ^{a}	14.93 ± 1.01 ^{a}	22.11 ± 3.03 ^{ab}	30.53 ± 5.29 ^{b}	40.13 ± 3.62 ^{c}	<0.001 *
2-phenylethanol	12.84 ± 1.38 ^{c}	12.47 ± 4.44 ^{bc}	8.05 ± 0.47 ^{abc}	6.97 ± 1.20 ^{ab}	5.76 ± 0.22 ^{a}	0.007 *
methanol	5.36 ± 4.65	2.62 ± 0.45	3.44 ± 0.50	4.87 ± 0.73	3.46 ± 0.95	0.091 **
3-ethoxy-1-propanol	0.08 ± 0.01	0.15 ± 0.11	0.08 ± 0.01	0.10 ± 0.03	0.08 ± 0.01	0.773 **
3-(methylthio)-1-propanol	0.06 ± 0.01	0.07 ± 0.03	0.06 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	0.089 **
Total	**248.21 ± 7.96**	**199.93 ± 11.77**	**212.75 ± 11.05**	**229.68 ± 15.49**	**266.75 ± 11.06**	
Esters (mg/L)						
ethyl acetate	27.15 ± 0.80 ^{a}	25.02 ± 1.67 ^{a}	23.58 ± 1.97 ^{a}	27.21 ± 3.17 ^{a}	35.19 ± 2.14 ^{b}	<0.001 *
isoamyl acetate	1.03 ± 0.09 ^{b}	1.02 ± 0.27 ^{ab}	0.47 ± 0.08 ^{a}	0.21 ± 0.01 ^{a}	0.23 ± 0.02 ^{a}	<0.001 **
2-phenylethyl acetate	0.60 ± 0.06 ^{b}	0.50 ± 0.15 ^{ab}	0.15 ± 0.03 ^{a}	0.06 ± 0.01 ^{a}	0.06 ± 0.00 ^{a}	0.001 **
ethyl octanoate	0.48 ± 0.09 ^{ab}	0.54 ± 0.12 ^{ab}	0.23 ± 0.02 ^{b}	0.14 ± 0.03 ^{ab}	0.10 ± 0.02 ^{a}	0.002 **
ethyl hexanoate	0.34 ± 0.07 ^{ab}	0.27 ± 0.07 ^{ab}	0.12 ± 0.01 ^{b}	0.07 ± 0.02 ^{ab}	0.05 ± 0.01 ^{a}	0.004 **
ethyl decanoate	0.30 ± 0.10 ^{ab}	0.29 ± 0.06 ^{ab}	0.10 ± 0.01 ^{b}	0.04 ± 0.01 ^{a}	0.022 ± 0.003 ^{a}	0.002 **
ethyl butyrate	0.12 ± 0.03	0.07 ± 0.01	0.05 ± 0.01	0.08 ± 0.05	0.10 ± 0.05	0.064 **
ethyl dodecanoate	0.07 ± 0.02	0.07 ± 0.02	0.007 ± 0.002	tr.	tr.	---
ethyl lactate	0.023 ± 0.003	0.03 ± 0.02	0.020 ± 0.005	0.017 ± 0.008	0.013 ± 0.004	0.257 **
ethyl phenylacetate	0.004 ± 0.001	0.003 ± 0.000	0.003 ± 0.001	0.004 ± 0.002	0.003 ± 0.000	0.682 **
Total	**30.13 ± 0.83**	**27.81 ± 1.71**	**24.72 ± 1.97**	**27.83 ± 3.17**	**35.76 ± 2.14**	
Volatile phenols (µg/L)						
4-vinylphenol	195.17 ± 29.68 ^{ab}	178.63 ± 49.50 ^{ab}	144.72 ± 6.20 ^{a}	96.49 ± 17.00 ^{ab}	112.61 ± 8.72 ^{b}	0.016 **
4-vinylguaiacol	100.67 ± 9.17 ^{c}	85.13 ± 11.68 ^{bc}	67.52 ± 3.85 ^{ab}	50.92 ± 15.19 ^{a}	55.02 ± 5.31 ^{a}	0.001 *
Total	**295.84 ± 31.07**	**263.76 ± 50.86**	**212.24 ± 7.30**	**147.41 ± 22.80**	**167.63 ± 10.21**	

Table 3.3. (Cont.) Concentration of volatile compounds in meads obtained after fermentation by *S. cerevisiae* QA23 at different pitching rates. Pitching rates: (PR1) 1.5×10^5 CFUs/mL, (PR2) 10^6 CFUs/mL, (PR3) 10^7 CFUs/mL, (PR4) 4×10^7 CFUs/mL and (PR5) 10^8 CFUs/mL. Data are the means of triplicate fermentations ± S.D.

	PR1	PR2	PR3	PR4	PR5	*P*-value
Volatile fatty acids (µg/L)						
octanoic acid	2158.77 ± 124.05 ^{c}	1622.81 ± 509.27 ^{abc}	852.45 ± 118.22 ^{b}	516.75 ± 174.10 ^{ab}	308.85 ± 45.82 ^{a}	<0.001 **
decanoic acid	1028.31 ± 339.35 ^{ab}	540.88 ± 160.01 ^{ab}	222.08 ± 23.05 ^{b}	82.29 ± 42.77 ^{ab}	27.93 ± 6.04 ^{a}	0.001 **
hexanoic acid	600.66 ± 78.68 ^{c}	567.37 ± 202.10 ^{abc}	272.35 ± 28.96 ^{b}	155.67 ± 37.14 ^{ab}	118.25 ± 7.04 ^{a}	0.002 **
isobutyric acid	24.99 ± 11.44 ^{ab}	33.88 ± 20.73 ^{a}	44.15 ± 9.73 ^{ab}	102.77 ± 31.71 ^{ab}	213.19 ± 45.47 ^{b}	0.013 **
dodecanoic acid	55.39 ± 28.47	21.91 ± 11.43	17.40 ± 1.79	17.60 ± 1.26	15.95 ± 3.64	0.452 **
butanoic acid	16.90 ± 4.40 ^{ab}	20.24 ± 8.75 ^{ab}	12.04 ± 179 ^{a}	15.50 ± 3.82 ^{ab}	26.84 ± 4.35 ^{b}	0.047 *
Total	**3885.02 ± 371.08**	**2807.08 ± 571.35**	**1420.48 ± 124.28**	**890.58 ± 185.85**	**711.00 ± 65.46**	
Carbonyl compounds (mg/L)						
Acetaldehyde	7.12 ± 2.38	6.27 ± 0.49	9.81 ± 3.00	7.91 ± 2.83	7.53 ± 1.28	0.429 *

tr.- traces. a–d Means within a line with different superscripts differ, $P < 0.05$. Lack of a superscript indicates no significant difference, $P > 0.05$.

* *P*-values are those for the effect of pitching rate on the volatile profile of mead, from one-way ANOVA analysis. If there was a significant effect of pitching rate on the volatile compounds data, then the means were compared with Tukey's test because equal variances could be assumed ($P > 0.05$ by means of the Levene test).

** *P*-values are those for the effect of pitching rate on the volatile profile of mead, from one-way Welch ANOVA analysis. If there was a significant effect of pitching rate on the volatile compounds data, then the means were compared with the Dunnett T3's test because equal variances could not be assumed ($P < 0.05$ by means of the Levene test).

Table 3.4. Concentration of volatile compounds in meads obtained after fermentation by *S. cerevisiae* ICV D47 at different pitching rates. Pitching rates: (PR1) 1.5×10^5 CFUs/mL, (PR2) 10^6 CFUs/mL, (PR3) 10^7 CFUs/mL, (PR4) 4×10^7 CFUs/mL and (PR5) 10^8 CFUs/mL. Data are the means of triplicate fermentations ± S.D.

	PR1	PR2	PR3	PR4	PR5	*P*-value
Alcohols (mg/L)						
3-methyl-1-butanol	150.34 ± 28.98	139.83 ± 9.45	126.24 ± 14.83	146.00 ± 2.47	165.18 ± 13.76	0.236 **
2-methyl-1-propanol	19.86 ± 1.32 ^{a}	20.68 ± 1.19 ^{a}	24.65 ± 4.20 ^{a}	41.70 ± 3.27 ^{b}	74.73 ± 17.30 ^{ab}	0.002 **
2-methyl-1-butanol	21.28 ± 4.52 ^{a}	19.86 ± 2.32 ^{a}	23.93 ± 6.06 ^{a}	34.67 ± 2.96 ^{b}	35.54 ± 2.84 ^{b}	0.001 *
1-propanol	18.66 ± 1.49 ^{a}	22.76 ± 2.47 ^{a}	32.62 ± 0.97 ^{b}	36.60 ± 5.99 ^{b}	52.53 ± 2.02 ^{c}	< 0.001 *
2-phenylethanol	12.68 ± 1.03 ^{b}	11.07 ± 0.45 ^{b}	7.62 ± 1.19 ^{a}	7.86 ± 0.80 ^{a}	7.95 ± 1.55 ^{a}	< 0.001 *
methanol	4.08 ± 0.04	4.67 ± 0.85	8.94 ± 7.69	18.40 ± 25.28	5.48 ± 1.85	0.545 **
3-(methylthio)-1-propanol	0.09 ± 0.01 ^{ab}	0.07 ± 0.01 ^{a}	0.07 ± 0.03 ^{a}	0.15 ± 0.04 ^{b}	0.24 ± 0.04 ^{c}	< 0.001 *
3-ethoxy-1-propanol	0.004 ± 0.001	0.010 ± 0.002	0.007 ± 0.001	0.008 ± 0.003	0.009 ± 0.003	0.080 *
Total	**226.98 ± 29.42**	**218.95 ± 10.15**	**224.07 ± 18.33**	**285.38 ± 26.48**	**341.64 ± 22.51**	
Esters (mg/L)						
ethyl acetate	22.73 ± 1.21	25.76 ± 4.18	21.36 ± 1.40	17.91 ± 3.25	20.24 ± 3.39	0.074 *
isoamyl acetate	1.34 ± 0.24 ^{ab}	1.26 ± 0.15 ^{b}	0.56 ± 0.14 ^{a}	0.23 ± 0.02 ^{a}	0.18 ± 0.02 ^{a}	0.001 **
2-phenylethyl acetate	0.69 ± 0.15 ^{ab}	0.57 ± 0.03 ^{b}	0.14 ± 0.03 ^{a}	0.08 ± 0.01 ^{a}	0.08 ± 0.01 ^{a}	< 0.001 **
ethyl octanoate	0.39 ± 0.09 ^{ab}	0.44 ± 0.06 ^{ab}	0.32 ± 0.03 ^{b}	0.23 ± 0.01 ^{ab}	0.172 ± 0.003 ^{a}	0.003 **
ethyl hexanoate	0.21 ± 0.03 ^{bc}	0.23 ± 0.01 ^{c}	0.17 ± 0.05 ^{abc}	0.095 ± 0.003 ^{ab}	0.08 ± 0.01 ^{a}	< 0.001 **
ethyl decanoate	0.11 ± 0.02 ^{ab}	0.14 ± 0.04 ^{b}	0.09 ± 0.03 ^{ab}	0.05 ± 0.02 ^{a}	0.04 ± 0.01 ^{a}	0.005 *
ethyl butyrate	0.07 ± 0.01	0.12 ± 0.07	0.06 ± 0.02	0.07 ± 0.03	0.06 ± 0.05	0.738 **
ethyl lactate	0.02 ± 0.01	0.022 ± 0.004	0.019 ± 0.004	0.017 ± 0.006	0.013 ± 0.001	0.121 *
ethyl dodecanoate	0.01 ± 0.02	0.04 ± 0.01	tr.	tr.	tr.	---
ethyl phenylacetate	0.003 ± 0.001	0.004 ± 0.000	0.003 ± 0.001	0.003 ± 0.001	0.013 ± 0.001	0.231 *
Total	**25.59 ± 1.24**	**28.57 ± 4.19**	**22.73 ± 1.41**	**18.68 ± 3.25**	**20.87 ± 3.39**	
Volatile phenols (µg/L)						
4-vinylphenol	160.07 ± 2.49 ^{bd}	166.24 ± 13.53 ^{d}	155.31 ± 21.32 ^{cd}	74.55 ± 0.84 ^{ac}	67.80 ± 21.90 ^{ab}	< 0.001 **
4-vinylguaiacol	89.39 ± 10.15 ^{c}	103.00 ± 18.33 ^{abc}	87.44 ± 14.35 ^{bc}	31.23 ± 1.15 ^{ab}	29.83 ± 7.12 ^{a}	0.003 **
Total	**249.46 ± 10.45**	**269.24 ± 22.78**	**242.76 ± 25.70**	**105.78 ± 1.42**	**97.63 ± 23.03**	

Table 3.4. (Cont.) Concentration of volatile compounds in meads obtained after fermentation by *S. cerevisiae* ICV D47 at different pitching rates. Pitching rates: (PR1) 1.5×10^5 CFUs/mL, (PR2) 10^6 CFUs/mL, (PR3) 10^7 CFUs/mL, (PR4) 4×10^7 CFUs/mL and (PR5) 10^8 CFUs/mL. Data are the means of triplicate fermentations ± S.D.

	PR1	PR2	PR3	PR4	PR5	*P*-value
Volatile fatty acids (µg/L)						
octanoic acid	1344.51 ± 239.84	1869.02 ± 903.08	901.17 ± 226.76	657.89 ± 197.15	441.05 ± 54.43	0.020 **
hexanoic acid	420.61 ± 27.85 c	448.50 ± 19.92 c	308.74 ± 12.43 b	193.94 ± 57.86 ab	167.74 ± 11.50 a	< 0.001 **
decanoic acid	401.86 ± 62.45 c	374.05 ± 66.56 c	224.95 ± 69.15 b	108.92 ± 38.57 ab	49.16 ± 12.08 a	< 0.001 *
isobutyric acid	21.95 ± 5.39	36.29 ± 1.87	61.55 ± 16.99	140.68 ± 35.17	270.70 ± 114.89	0.017 **
dodecanoic acid	16.23± 5.46	15.92 ± 4.65	15.29 ± 7.29	15.88 ± 5.77	17.10 ± 4.89	0.996 *
butanoic acid	14.78 ± 4.57	17.29 ± 2.06	17.70 ± 3.43	16.44 ± 4.41	14.26 ± 2.23	0.694 *
Total	**2219.94 ± 249.55**	**2761.06 ± 905.77**	**1529.40 ± 238.13**	**1133.74 ± 212.11**	**960.00 ± 128.33**	
Carbonyl compounds (mg/L)						
Acetaldehyde	6.43 ± 2.30	6.54 ± 0.61	8.00 ± 0.72	8.15 ± 1.04	9.16 ± 1.22	0.131 *

tr. – traces. a–d Means within a line with different superscripts differ, $P < 0.05$. Lack of a superscript indicates no significant difference, $P > 0.05$.

* *P*-values are those for the effect of pitching rate on the volatile profile of mead, from one-way ANOVA analysis. If there was a significant effect of pitching rate on the volatile compounds data, then the means were compared with Tukey's test because equal variances could be assumed ($P > 0.05$ by means of the Levene test).

** *P*-values are those for the effect of pitching rate on the volatile profile of mead, from one-way Welch ANOVA analysis. If there was a significant effect of pitching rate on the volatile compounds data, then the means were compared with the Dunnett T3's test because equal variances could not be assumed ($P < 0.05$ by means of the Levene test).

Meads obtained with different pitching rates and fermented by the two strains showed quantitative differences in aroma profiles, confirming the contribution of both yeast metabolism and inoculum size on the sensory characteristics of meads. In general, the total concentration of volatile compounds increased with increasing pitching rate, except for the lowest pitching rate (1.5×10^5 CFUs/mL) and was higher in meads inoculated with strain ICV D47.

Alcohols were quantitatively the most abundant volatile compounds in all of the meads, confirming the importance of this group of volatile compounds produced by yeast during alcoholic fermentation (Ugliano and Henschke, 2009). Overall, we observed that increasing inoculum size led to higher concentration of alcohols. Our results are in agreement with those of Mateo et al. (2001) and Verbelen et al. (2009a, b), who studied the influence of yeast inoculum size on the fermentation performance and the volatile compound formation of wine and beer. The concentration of alcohols was below 300 mg/L in all of our meads, representing values considered desirable for increasing the complexity of wines (Ugliano and Henschke, 2009; Mateo et al., 2001). Quantitatively, the major alcohol in all of the meads was 3-methyl-1-butanol (Tables 3.3 and 3.4). There are few studies of mead aroma composition; however, our results are in accord with those of Mendes-Ferreira et al. (2010), who verified that the alcohol 3-methyl-1-butanol was the major compound quantified in mead obtained with the same formulation used in our work, at a concentration of approximately 140 mg/L. Similar concentrations of this compound were obtained in our work, irrespective of the yeast strain used. The concentrations of 1-propanol, 2-methyl-1-propanol and 2-methyl-1-butanol increased with increasing pitching rates, except for the two lowest inoculum sizes (1.5×10^5 CFUs/mL and 10^6 CFUs/mL). Verbelen et al. (2008), working in brewing, also detected an increase in higher alcohol formation when using high pitching rates. No differences in 3-methyl-1-butanol were observed in meads fermented at different pitching rates by the strain ICV D47, whereas a higher concentration of this alcohol was detected only at the lowest pitching rate (1.5×10^5 CFUs/mL) with the strain QA23. Different results were obtained by Mateo et al. (2001), Verbelen et al. (2008) and Verbelen et al. (2009a), who indicated a direct dependence of the concentration of that compound on inoculum size.

Esters represented the most diverse group with ten compounds quantified. Their concentrations varied between 18 and 35 mg/L, with the highest ester concentrations found in the meads fermented by the strain QA23. No clear trend was observed between the total concentration of esters and the pitching rate, although minor differences were observed

among meads fermented at different pitching rates. Ethyl acetate was the major ester compound quantified, although at lower concentrations than those detected by Mendes-Ferreira et al. (2010) for the same alcoholic beverage. Similar concentrations were detected by Verbelen et al. (2009a) in beer. Other authors showed that ester levels were negatively influenced by higher pitching rates (Verbelen et al., 2008). A similar result was observed in this study for isoamyl acetate and 2-phenylethyl acetate, compounds with fruity and flowery flavours, respectively. Also Erten et al. (2006) found an inverse correlation between inoculum size and the concentration of isoamyl acetate. In fact, the highest concentration of the ester was found in wines fermented with the lowest cell density (10^5 cells/mL).

Volatile phenols are predominantly produced by yeast during fermentation and are known for their contribution to off-flavours (Swiegers et al., 2005). Two phenols and in particular 4-vinylphenol, were identified in meads at concentrations below their respective detection thresholds. There were no relevant differences between the two strains with respect to these compounds; however, increasing the pitching rate resulted in a slight decrease in their concentration.

The most abundant of six volatile fatty acids (VFA) quantified was octanoic acid and the amount of this compound was independent of the yeast strain. In general, the concentration of VFA decreased with increasing pitching rate, except for the strain ICV D47. At the lowest pitching rates, 1.5×10^5 CFUs/mL and 10^6 CFUs/mL, the strain QA23 produced more VFA than did the strain ICV D47, whereas the opposite was observed at higher pitching rates. Two of the six compounds quantified, hexanoic and octanoic acids, were above their respective detection thresholds. The results obtained here are in agreement with those of Mendes-Ferreira et al. (2010), who verified that octanoic acid was quantitatively the major volatile fatty acid founds in meads, followed by hexanoic and decanoic acids. Acetaldehyde was the only carbonyl compound quantified in meads obtained after fermentation with strains QA23 and ICV D47. This compound is quantitatively the most important saturated aldehyde produced from sugar metabolism and ranges in a concentration from 10 to 75 mg/L (Swiegers et al., 2005). Acetaldehyde formation is known to be highly variable among strains of *S. cerevisiae* (Ugliano and Henschke, 2009), although in our study, both strains produced similar concentrations of this aldehyde. Some non-significant variation in acetaldehyde formation was observed among pitching rates. No relationship between the concentration of acetaldehyde and the inoculum size was observed for strain QA23, corroborating the results previously obtained by Erten et al. (2006) who had found no effect on this or other carbonyl

compounds. On the contrary, ICV D47 formed less acetaldehyde at high pitching rates, as observed by Verbelen et al. (2009a).

In previous studies of the influence of volatile compounds on wine aroma, the Odour Activity Values (OAVs) were determined (Escudero et al., 2004; Vilanova et al., 2009; Vilanova et al., 2010). To evaluate the contribution of a certain chemical compound to the aroma of mead, the OAVs were calculated by dividing the concentration of each compound by its perception threshold. Only those compounds whose OAV was greater than 1 were considered to cause a significant contribution to the mead's aroma. It should be pointed out that individual OAVs do not account for the antagonistic or synergistic effects resulting from the perceptual interactions between different molecules present in wines, but they can serve as estimates for the potential contribution of each compound to the global aroma (Vilanova et al., 2009). Those compounds, including odour descriptors and thresholds, are displayed in Table 3.5.

Fourteen of the twenty-seven volatile compounds quantified could have a valuable contribution to mead's aroma and flavour, because their concentrations were above their corresponding thresholds. The most aromatic meads were dependent not only on the fermentative strain but also on the pitching rate tested. More aromatic meads were fermented by *S. cerevisiae* strain QA23 at small inoculum sizes (1.5×10^5 CFUs/mL and 10^6 CFUs/mL). By contrast, the strain ICV D47 produced aroma compounds with higher OAVs than QA23 at high pitching rates (10^7 CFUs/mL to 10^8 CFUs/mL). However, at low pitching rates, more interesting aroma compounds were released by both strains. In general, the lower the pitching rate, the higher the OAVs of the resulting mead, except for the mead fermented by ICV D47 at 10^6 CFUs/mL.

Ethyl hexanoate, ethyl octanoate and isoamyl acetate were the most powerful odourants detected in all of the meads. In fact, commercial wine strains produce variable amounts of esters, such as isoamyl acetate, ethyl hexanoate and ethyl octanoate, which have a potential impact on the aroma profile (Swiegers et al., 2005). Esters contribute favourably to aroma as a fruity characteristic. Indeed, ethyl octanoate and isoamyl acetate were two of the most abundant odourant compounds identified in the different meads. The OAV values of these compounds and of ethyl hexanoate decreased with increasing pitching rates.

Table 3.5. Odour activity values (OAV) of volatile compounds of more influence on the aroma of meads obtained after fermentation by *S. cerevisiae* QA23 and *S. cerevisiae* ICV D47 at different pitching rates. Pitching rates: (PR1) 1.5×10^5 cells/mL, (PR2) 10^6 cells/mL, (PR3) 10^7 cells/mL, (PR4) 4×10^7 cells/mL and (PR5) 10^8 cells/mL.

			QA 23					ICV D47				
Compounds	**Odor descriptor [a]**	**Odor threshold (µg/L) [a]**	**PR1**	**PR2**	**PR3**	**PR4**	**PR5**	**PR1**	**PR2**	**PR3**	**PR4**	**PR5**
3-methyl-1-butanol	Cheese; nail polish	30 000	5.60	4.45	4.28	3.92	4.10	5.01	4.66	4.21	4.87	5.51
2-methyl-1-propanol	Alcohol; bitter	40 000	---	---	---	1.03	1.56	---	---	---	1.04	1.87
ethyl acetate	Solvent like; nail polish	12 300	2.21	2.03	1.92	2.21	2.86	1.85	2.09	1.74	1.46	1.65
isoamyl acetate	Banana	30	34.43	34.16	15.59	7.06	7.53	44.75	42.09	18.57	7.59	6.16
2-phenylethyl acetate	Flowery; roses	250	2.40	2.02	---	---	---	2.75	2.28	---	---	---
ethyl octanoate	Fruity; sweet	5	95.44	107.11	46.47	27.19	19.17	78.72	87.48	63.21	45.03	34.48
ethyl hexanoate	Fruity; aniseed	14	24.64	19.12	8.28	5.10	3.78	15.17	16.27	12.01	6.79	5.56
ethyl decanoate	Pleasant; soap	200	1.52	1.43	---	---	---	---	---	---	---	---
ethyl butyrate	Fruity; pineapple	20	6.22	3.39	2.41	3.90	5.20	3.74	5.81	2.92	3.36	2.97
4-vinylphenol	Almond shell	180	1.08	---	---	---	---	---	---	---	---	---
octanoic acid	Fatty; rancid	500	4.32	3.25	1.70	1.03	---	2.69	3.74	1.80	1.32	---
decanoic acid	Fatty; soapy	1000	1.03	---	---	---	---	---	---	---	---	---
hexanoic acid	Cheese; sweaty	420	1.43	1.35	---	---	---	1.00	1.07	---	---	---
acetaldehyde	Fresh; green leaves	500	14.24	12.55	19.61	15.81	15.06	12.86	13.08	15.99	16.31	18.32
Total			**195.11**	**192.89**	**103.73**	**68.71**	**61.15**	**170.87**	**181.08**	**123.90**	**89.33**	**78.70**

[a] Odor descriptors and odor threshold reported in the literature (Guth, 1997, Moreno *et al*, 2005, Siebert *et al*, 2005, Culleré *et al*, 2004, Escudero *et al*, 2004, Ferreira *et al*, 2000, Boidron *et al*., 1988, Czerny *et al*., 2008).

Scientific studies of mead production and quality are limited and are mainly concerned with the selection of yeasts for inoculation of honey-musts and with the impact of honey-must formulation on mead quality. This is the first study of the effects of inoculum size on the optimisation of mead production and final quality. Our results demonstrate that increasing pitching rates results in significant time savings in the fermentation process. However, caution should be taken, as an exaggerated inoculum could lead to lower production of desirable aromatic compounds. In addition to this quantitative analysis of the impact of strain selection and inoculum size on mead aroma, a complementary sensorial evaluation of the meads would yield further useful information for mead producers.

CHAPTER 4

Mead production: fermentative performance of yeasts entrapped in different concentrations of alginate

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Abstract

Mead is an alcoholic drink known since ancient times, produced by yeast fermenting diluted honey. However, the production of mead has suffered in recent years, partially owing to the lack of scientific progress in this field. In this study, two strains of *Saccharomyces cerevisiae*, QA23 and ICVD47, were immobilized in 2 or 4% (w/v) alginate beads to assess the most effective alginate concentration for yeast immobilization to produce mead. Neither of the alginate concentrations was able to prevent the cell leakage from the beads. The fermentation length was 120 h for both yeast strains. In all cases, at the end of fermentation, the number of cells entrapped in the beads was higher than the number of free cells, and the total 4% alginate bead wet weight was significantly higher than the 2% alginate bead wet weight. In addition, the evaluation of mead quality showed that the yeast strain had significantly more influence on the physicochemical characteristics than the alginate concentration. Although the yeasts immobilized in the two alginate concentrations were able to perform the fermentation, further research is needed in order to understand the evolution of the yeast population inside the beads throughout the fermentative process.

Keywords: alginate concentration, cell leakage, mead, yeast immobilization.

Introduction

Mead has been produced since ancient times, mainly in an empirical and artisanal manner. This drink has been reported to contain many of the elements required by humans and to have excellent effects on digestion and metabolism. It has also been considered to be beneficial for people who suffer from chronic anaemia and diseases of the gastrointestinal tract (Gupta and Sharma, 2009). Mead, which results from the fermentation of diluted honey, can have an alcoholic content that ranges from 8 to 18% (v/v). This is accomplished by varying the proportions of honey and water and the point at which the fermentation is stopped (Ramalhosa et al., 2011). The fermentative process and maturation require an extended period in which several problems may occur. For instance, the anticipated alcohol content may not be achieved, a successive addition of honey may be needed to avoid the premature end of fermentation, and there is a high likelihood for stuck fermentations (Ramalhosa et al., 2011). This is related to the specific properties of the honey solution, mainly the high sugar concentration, high acidity, low protein content, low indigenous microbiota and the shortage of substances essential for yeast development (Sroka and Tuszyński, 2007).

Indeed, this complex fermentative process depends on several factors, such as the type of honey, yeast strain, honey-must composition and pH (Navrátil et al., 2001). In the past few years, several studies on the optimization of mead production have been carried out, mainly regarding yeast selection and honey -must formulation (Mendes-Ferreira et al., 2010; Pereira et al., 2009; Pereira et al., 2013; Sroka and Tuszyński, 2007). However, it is worth nothing that immobilized cells were used in just two of the studies involving mead production (Navrátil et al., 2001; Qureshi and Tamhane, 1986).

The application of immobilized yeast cells for the production of alcoholic beverages has been extensively studied in the past few years. Cell immobilization has some advantages over free cells, such as high cell loads, high volumetric productivities, increased substrate uptake, protection from inhibitory substances and reuse of the same biocatalyst for extended periods of time (Bezbradica et al., 2007; Kourkoutas et al., 2004; Park and Chang, 2000; Tsakiris et al., 2004; Vilela et al., 2013). One of the most common methods of immobilization is the entrapment of cells in hydrogels, which involves entrapping living cells within a rigid network, which permits the diffusion of substrates and products, thereby making possible cell growth and the maintenance of active cells (Divies and Cachon, 2005). Calcium alginate gels have been the most widely used matrices for cell entrapment owing to their simplicity (Inal

and Yiğitoğlu, 2011). Alginate is a natural co-polymer that is gelled when it comes into contact with bivalent cations such as Ca^{2+}, forming beads (Liouni et al., 2008). Despite its numerous advantages, some problems can occur in an alcoholic fermentation using yeast cells entrapped in Ca-alginate beads, the most common being cell leakage. This phenomenon results in destabilization and rupture of beads, mainly owing to cell growth, and gas formation and accumulation within the beads, as well as the presence of chelators in the medium (Liouni et al., 2008). The aim of the present study was to investigate the capacity of two sodium alginate concentrations, 2 and 4%, to immobilize *Saccharomyces cerevisiae* yeast strains QA23 and ICV D47, in the context of mead production. The cells were entrapped in the gels by a drop-forming procedure and with the goal of evaluating the most effective alginate concentration.

Material and Methods

Yeast strains

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

Honey

A dark honey was purchased from a local beekeeper in the northwest of Portugal. A palynological analysis of the honey was performed according to the acetolytic method (Pires et al., 2009) and it was determined that this multifloral honey was derived primarily from the pollen of *Castanea* spp. (45%) and *Erica* spp. (32%). The characteristics and satisfactory quality of the honey were in agreement with the requirements established by Portuguese legislation (Decreto-Lei nº 214/2003, 18 September).

Preparation of honey-must for fermentation

The honey-must for fermentation was prepared as described by Pereira et al. (2013). Honey was diluted (to 37% w/v) using natural spring-water to obtain, at the end of fermentation, an alcoholic beverage of approximately 11% of ethanol, with the solution mixed to homogeneity. Insoluble materials were removed from the mixture by centrifugation (2682×*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,

USA) and pH was adjusted to 3.7 with malic acid (Merck, Darmstadt, Germany). The nitrogen content was adjusted to 267 mg/L with diammonium phosphate (DAP, BDH Prolabo, Leuven, Belgium). The parameters ºBrix, pH, titatrable acidity and assimilable nitrogen concentration were determined, prior and after the adjustments. The honey-must was pasteurised at 65 ºC for 10 min and then immediately cooled. No sulphur dioxide was added to the honey- musts.

Immobilization of yeast cells

The dry yeast was hydrated by dissolving 2 g of active dry yeast in 20 mL of sterilized water at 38 ºC, according to the manufacturer's instructions, to obtain 10^8 colony forming units (CFU)/mL. Sodium alginate (BDH Prolabo, Leuven, Belgium) was dissolved in distilled water at concentrations of 2 and 4% (w/v). The calcium chloride (Panreac, Barcelona, Spain) solution was prepared with distilled water at a concentration of 180 mM. Sodium alginate and calcium chloride solutions were autoclaved at 121 ºC for 15 min, and then were cooled. To inoculate the honey-must with 10^6 CFUs/mL, the appropriate amount of yeast suspension was added to 10 mL of a sodium alginate solution. The polymer–cell mixture was added dropwise to the $CaCl_2$ solution and left to harden in this solution for 30 min at 4ºC. The *S. cerevisiae* immobilized beads were rinsed three times with sterile distilled water, and then transferred into the honey-must.

Fermentation conditions and monitoring

The immobilized beads were transferred into the honey-must for batch fermentation. All fermentations were carried out using a previously described system (Mendes-Ferreira et al., 2010), 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. The flasks were maintained during alcoholic fermentation at 25 °C under continuous, but moderate shaking (120 rpm), mimicking an industrial environment. Aseptic sampling for monitoring the fermentation was performed using a syringe-type system as previously described (Mendes-Ferreira et al., 2009). Fermentations were monitored daily by weight loss as an estimate of CO_2 production. For determining the growth parameters of suspended cells in the medium, samples were collected and appropriately diluted for the measurement of their optical density at 640 nm in a UV–VIS spectrometer (Unicam Helios) and for counting CFU in solid Yeast Peptone Dextrose agar (YPD – 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. At the end of the alcoholic fermentation, samples were taken from the fermented media for several analytical determinations.

Analyses performed at the end of fermentation

At the end of fermentation, the culture dry weight of the suspended cells in the medium 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. For determination of dry weight, determination of the concentration of viable cells immobilized in the beads and the immobilization yield at the end of fermentation, the beads were liquefied using a chemical method, according to a procedure adapted from Göksungur and Zorlu (2001). Fifty beads were washed with water, dissolved in 50 mL of a sterilized sodium citrate solution (50 mM), with continuous stirring for 30 min at room temperature. The dry weight of the immobilized cells was determined by the same procedure as described previously for suspended cells in medium. For assessing the growth of immobilized cells, after appropriate dilutions of liquefied beads, these were counted as the number of CFU in solid YPD plates, after incubation at 25 °C for 48 h. The immobilization yield was calculated as the immobilized dry weight of yeasts/immobilized and free dry weight of yeasts × 100 (Inal and Yiğitoğlu, 2011).

The oenological parameters, such as total sulphur dioxide (SO_2), pH, titratable acidity, volatile acidity and ethanol content, were determined according to standard methods (Organisation Internationale de la Vigne et du Vin, 2006). Yeast assimilable nitrogen (YAN) was determined by the formaldehyde method as previously described (Aerny, 1996). After clarification, 10 mL of the sample was transferred into a 50 mL beaker and diluted with 15 mL of water. The pH was adjusted to 8.1 with 100 mM NaOH and 2.5 mL of formaldehyde at pH 8.1 was added. After 5 min, the pH was adjusted again to 8.1 by titration with 50 mM NaOH. Assimilable nitrogen was calculated using the following formula:

$$\text{YAN (mg/L)} = [(\text{vol. NaOH}) \times (\text{conc. NaOH}) \times 14 \times 1000]/ (\text{sample volume})$$

Statistical analysis

All of the fermentation experiments were performed in duplicate and the results are expressed as mean values and standard deviation. The data were analysed using SPSS Software, version 17.0 (SPSS, Inc.). To test significant differences amongst the

physicochemical characteristics of meads and beads characteristics, a two factor – alginate concentration (A) and strain (S) – analysis of variance (ANOVA) was applied. In order to compare the means between two unrelated groups (2 and 4% of alginate) for each strain, an independent-samples t-test was performed. The fulfilment of the ANOVA requirements, namely the normal distribution of the residuals and the homogeneity of variance, were evaluated by means of the Shapiro–Wilks test ($n < 50$) and Levene's test, respectively. All statistical tests were performed at a 5 % significance level.

Results and Discussion

In this work the immobilization of *S. cerevisiae* yeast strains QA23 and ICV D47, using two alginate concentrations (2 and 4%), with a population corresponding to approximately 10^6 CFU/mL, was studied. The effectiveness of the immobilization was determined by counting the yeast cells released from the beads into the medium and by analysing the reducing sugar consumption profile (Figure 4.1). Minor differences were detected in the number of CFU in the medium and in reducing sugars of the fermentations carried out with the cells immobilized in 2 or 4 % of Ca-alginate, using both strains. The strain ICV D47 immobilized in 4% of Ca-alginate showed a slightly higher sugar consumption until 72 h of fermentation. Nevertheless, all fermentations ended after 120 h with similar concentrations of residual sugars, ranging from 15.13 ± 0.49 to 19.89 ± 2.57 g/L, in meads fermented by the strains ICV D47 and QA23, respectively, and entrapped in 2% alginate beads. Similar concentrations of residual sugars and times of fermentation were obtained for mead production using free yeast cells (Mendes-Ferreira et al., 2010; Pereira et al., 2013). These residual sugars include disaccharides such as sucrose, maltose, isomaltose, trisaccharides and tetrasaccharides (Pereira et al., 2013). The growth kinetics profile shows that, at the beginning of fermentation, the number of free cells in the medium was higher when yeast cells were entrapped in 2% than in 4% alginate. This difference was seen more clearly for strain QA23. For this strain, at the end of fermentation, the number of free cells in medium was 6.8×10^6 CFUs/mL and 4.8×10^6 CFUs/mL when immobilized in 2 and 4% Ca-alginate, respectively. For the strain ICV D47, minor differences were observed at the end of fermentation in the number of free cells in medium for both alginate concentrations (1.3×10^7 CFUs/mL). The

strain ICV D47, presented a higher number of free cells in medium. However for both strains, independent of the alginate concentration used, the number of cells in medium had increased to 10^6 – 10^7 CFU/mL in the first 48 h and then remained constant until the end of the fermentation. The evolution of CO_2, particularly in the first 48 h of fermentation, may cause an internal mechanical loading of the beads, leading to the disintegration of the majority of beads (Göksungur and Zorlu, 2001). The increase in the cell population in the medium was exponential, resulting from the combined effects of cell leakage from the beads and the proliferation of free cells in the medium. Other authors have obtained similar results 30 h after cultivation, when a different entrapment agent (LentiKat® carrier) was applied (Bezbradica et al., 2007).

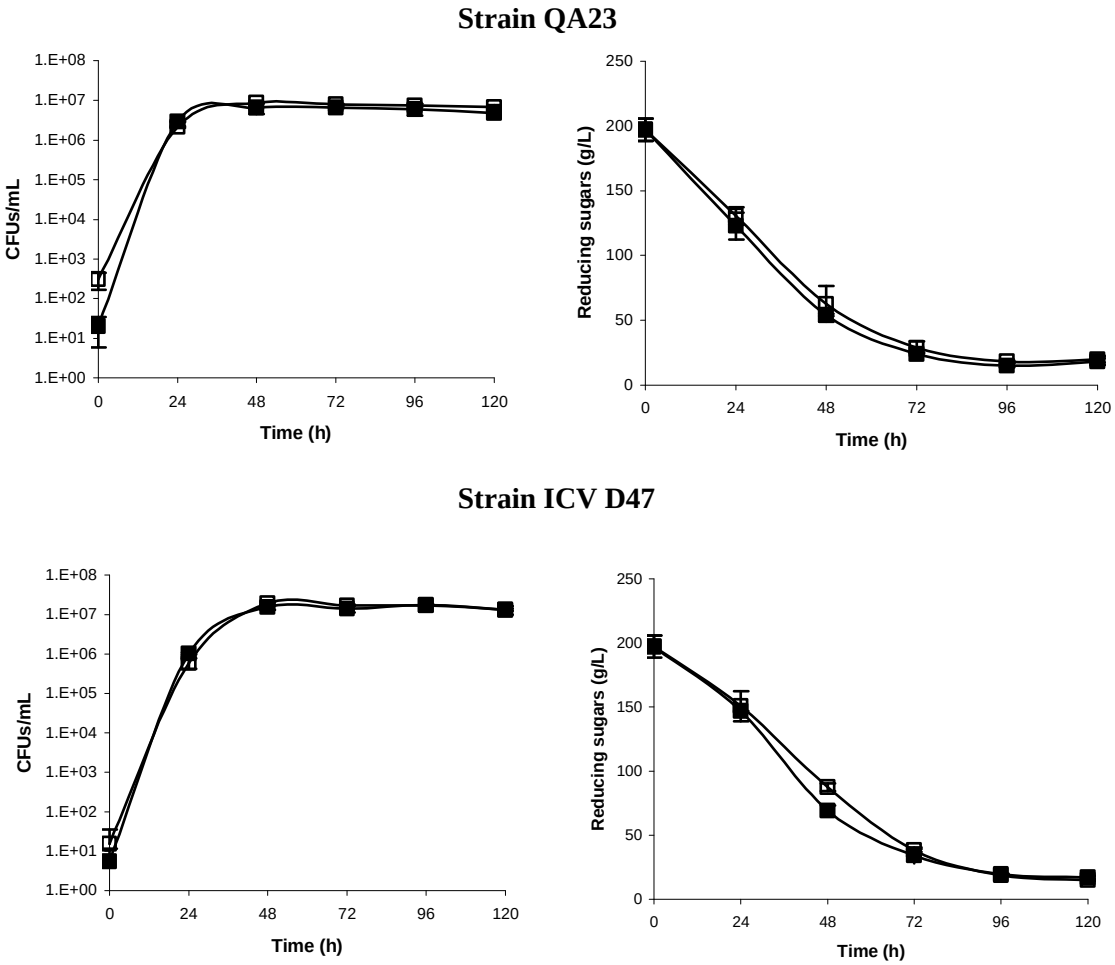

Figure 4.1. Growth of the free cells in medium and reduced sugar consumption by *Saccharomyces cerevisiae* QA23 and ICV D47 immobilized cells in 2% (□) and 4% (■) alginate.

Yeast cell growth was confirmed by cellular dry weight values and it was verified that the dry weight of cells in the beads was higher than that of the free cells in medium, irrespective of the concentration of Ca-alginate and the yeast strain used (Figure 4.2). This observation corroborates previous results obtained with *S. cerevisiae* encapsulated in polyvinyl alcohol particles for a beer fermentation (Bezbradica et al., 2007), in which a higher final cell concentration in the LentiKats® carrier than in suspended cells (4×10^8 cells/mL of carrier vs 3×10^7 cells/mL) was observed.

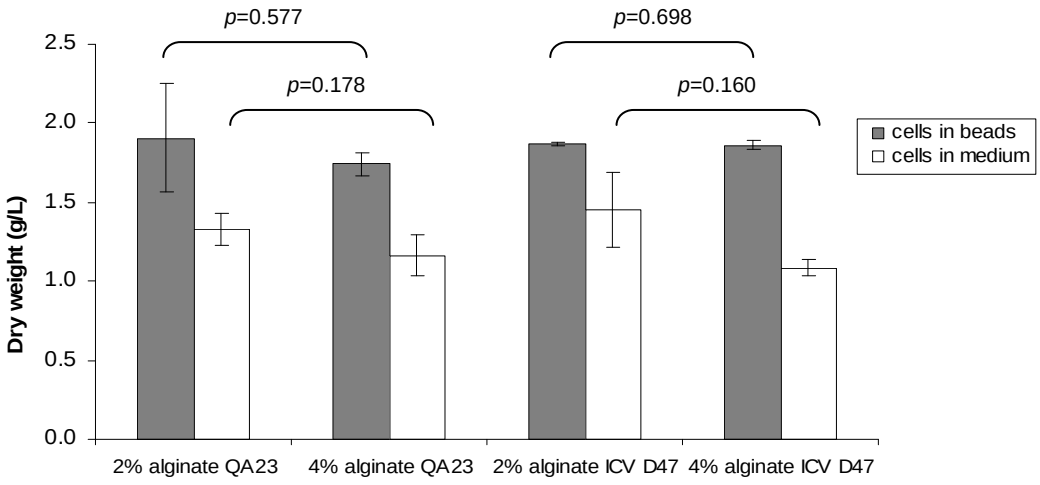

Figure 4.2. Cell dry weight of *S. cerevisiae* QA23 and ICV D47, at the end of fermentation, suspended in the medium (□) and inside the beads (■).

At the end of the fermentation, several growth parameters were determined after dissolution of the beads in a sodium citrate solution and these are presented in Table 4.1. The total wet weight of the 4% Ca-alginate beads was significantly higher when compared with the 2% Ca-alginate beads (p = 0.011 for QA23 and p = 0.015 for ICV D47). Significant differences were found in the total bead wet weight between the alginate concentrations (p < 0.001). No significant differences were detected in the number of CFU/mL between the strains or the alginate concentrations, with the lower value of $9.89 \pm 1.64 \times 10^7$ for mead produced using 2% alginate QA23 beads and the higher value of $1.88 \pm 0.07 \times 10^8$, corresponding to the mead produced using 4% alginate ICV D47 beads. Regarding the immobilization yield, although the values obtained with 4% alginate were higher for both strains, the differences between the two concentrations of alginate were not significant.

Table 4.1. Total beads wet weight, colony forming units (CFU) and immobilization yield of *Saccharomyces cerevisiae* QA23 and ICV D47 immobilized cells in 2 or 4% alginate.

Meads	Strain QA23		Strain ICV D47		Significance		
	2% alginate	4% alginate	2% alginate	4% alginate	Alginate (A)	Strain (S)	A*S
Total beads wet weight (g)	8.36 ± 0.25 *	10.15 ± 0.09 *	7.65 ± 0.33 *	11.10 ± 0.52 *	< 0.001	n.s.	0.025
CFUs/mL of alginate	1.40 ± 0.61 E+08	9.89 ± 1.64 E+07	1.82 ± 0.43 E+08	1.88 ± 0.07 E+08	n.s.	n.s.	n.s.
Immobilization yield (%)	58.73 ± 6.15	59.82 ± 3.79	56.43 ± 4.12	63.24 ± 1.45	n.s.	n.s.	n.s.

* significant difference between the alginate concentrations for each strain ($p < 0.05$); lack of superscript indicates no significant difference; n.s., no significant difference at $p < 0.05$.

Table 4.2. Physicochemical characteristics of honey-must and meads fermented by *S. cerevisiae* QA23 and ICV D47 immobilized cells in 2 or 4% alginate.

Honey-must	
pH	3.71 ± 0.00
º Brix (%)	23.20 ± 0.14
Titratable acidity $_{\text{tartaric acid}}$ (g/L)	3.43 ± 0.03
Initial nitrogen $_{\text{YAN}}$ (mg/L)	353.50 ± 4.95

Meads	Strain QA23		Strain ICV D47		Significance		
	2% alginate	4% alginate	2% alginate	4% alginate	Alginate (A)	Strain (S)	A * S
pH	3.66 ± 0.02	3.67 ± 0.03	3.62 ± 0.01	3.63 ± 0.01	n.s.	0.031	n.s.
Volatile acidity $_{\text{acetic acid}}$ (g/L)	0.63 ± 0.00	0.65 ± 0.02	0.51 ± 0.04	0.54 ± 0.04	n.s.	0.007	n.s.
Titratable acidity $_{\text{tartaric acid}}$ (g/L)	5.18 ± 0.00	5.14 ± 0.16	4.99 ± 0.21	5.10 ± 0.05	n.s.	n.s.	n.s.
Final nitrogen $_{\text{YAN}}$ (mg/L)	52.50 ± 4.95	42.00 ± 9.90	43.75 ± 2.47	45.50 ± 4.95	n.s.	n.s.	n.s.
Total SO_2 (mg/L)	23.68 ± 0.91	23.68 ± 0.91	21.12 ± 0.91	21.12 ± 0.91	n.s.	0.016	n.s.
Ethanol (% vol)	10.54 ± 0.94	11.20 ± 0.57	11.50 ± 0.14	11.40 ± 0.14	n.s.	n.s.	n.s.

Lack of superscript indicates no significant difference between the alginate concentrations for each strain ($p < 0.05$); n.s., no significant difference at $p < 0.05$

The quality of meads produced using strains QA23 and ICV D47 immobilized with 2 or 4% of Ca-alginate was assessed in terms of the physicochemical characteristics (pH, volatile acidity, titratable acidity, final nitrogen, total SO_2 and ethanol), and is presented in Table 4.2 The pH has been noted in the past as one of the causes of sluggish or premature fermentation arrest in alcoholic beverages (Mendes-Ferreira et al., 2010), which is why this parameter was determined in all of the experiments. As expected from previous work on mead production (Mendes-Ferreira et al., 2010; Pereira et al., 2013; Sroka and Tuszyński, 2007), the pH of the meads was lower than that of honey-musts (3.71 ± 0.00) for both strains and alginate concentrations. The reduction of pH during mead fermentation is probably caused by the production of acids by yeasts (Sroka and Tuszyński, 2007) and the low buffer capacity of honey-musts (Mendes-Ferreira et al., 2010). However, meads fermented by strain QA23 presented a significantly higher pH than those from strain ICV D47 (p = 0.031). Control of volatile acidity is a critical issue for the industrial manufacture of fermented beverages. Indeed, the production of acetic acid, by far the most abundant volatile acid, can have a dramatic effect on the quality of the final product. In addition to undesirable aromas, high levels of acetic acid are toxic to yeast and can lead to stuck alcoholic fermentations (Luo et al., 2013). The volatile acidity ranged from 0.51 ± 0.04 to 0.65 ± 0.02 g/L of acetic acid and was within the values reported for wine (Nikolaou et al., 2006) and the results obtained previously for mead produced without an immobilization systems (Mendes-Ferreira et al., 2010; Pereira et al., 2009; Pereira et al., 2013; Sroka and Tuszyński, 2007). For this parameter and for total SO_2, which varied between 21.12 ± 0.91 and 23.68 ± 0.91 mg/L, no differences were detected between meads obtained with either alginate concentrations. However, significant differences were observed between the strains (p = 0.007 for volatile acidity and p = 0.016 for total SO_2), with the lowest concentrations found in meads produced with strain ICV D47. Similar concentrations of titratable acidity, around 5 g/L of tartaric acid, were found in all meads irrespective of the strain or alginate concentration. Higher titratable acidity was found in meads, when compared with the honey-must, indicating the production of acids by the yeast. Different results were obtained during the fermentation of a fruit wine from cagaita, where a reduction of titratable acidity from 0.5% in must to 0.3% in wine was observed (Oliveira et al., 2011). The ethanol content ranged from 10.54 to 11.50 % (v/v) with no remarkable differences in meads fermented with immobilized cells in 2 or 4% Ca-alginate. Different results are reported in the literature. Najafpour et al. (2004) found that immobilization in 2% alginate was more suitable for ethanol production, based on the activity of the beads. Similar

amounts of ethanol have already been reported in fermentations of mead with the same initial ºBrix and produced with free cells (Mendes-Ferreira et al., 2010; Pereira et al., 2013). A concentration of residual nitrogen remained in all meads independent of the yeast strain and concentration of alginate used for immobilization. As reported previously, some of the residual nitrogen could correspond to the concentration of the amino acid proline, present in honey, which is not assimilated by yeast during the fermentation (Pereira et al., 2013).

In summary, at the end of the fermentation the number of cells entrapped in beads was higher than the number of free cells in the medium. Independent of the strain, the number of cells in the medium was similar for both concentrations of alginate. Considering the quality of meads, the results showed that the yeast strain had more influence than the concentration of alginate used for yeast entrapment. Indeed, the parameters of pH, volatile acidity and total SO_2 were significantly different between the two yeast strains.

Although the alginate concentrations tested did not prevent the phenomenon of cell leakage, the entrapment agent did not cause negative effects on mead production, since no remarkable differences were observed in fermentation performance and mead quality compared with mead produced previously with free cells. Fermentation length was 120 h and the characteristics of the final product were not influenced by the alginate concentration. Since no differences were found between the two alginate concentrations, for economic reasons using 2% of alginate for immobilization of yeasts for mead would be more advantageous. The current study also suggests that, considering the low volatile acidity produced by strain ICV D47, it appears be the more suitable yeast for immobilization.

CHAPTER 5

Effect of *Saccharomyces cerevisiae* cells immobilisation on mead production

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Abstract

Mead is a traditional alcoholic beverage obtained by the fermentation of diluted honey performed by yeasts. In this work the potential of application of immobilised yeast cells on single-layer Ca-alginate or double-layer alginate-chitosan for mead production was assessed for the first time. The meads produced either with entrapped or free cells were evaluated in terms of quality and aroma profile. The immobilisation procedure had no adverse effect on cell viability, since minor differences were found in fermentation kinetics among the strains and immobilisation systems. The double-layer alginate-chitosan had no advantage compared with the single-layer Ca-alginate, as the number of free cells in the medium, resulting from cell leakage, was similar. Although meads obtained with entrapped yeast cells presented less ethanol and glycerol and more acetic acid, it exhibited larger amounts of volatile compounds. Immobilised cells produced meads with more compounds with fruity characteristics, such as ethyl octanoate and ethyl hexanoate; however the concentrations of undesirable compounds in such meads were also higher. The effect of immobilisation on the aroma profile was important, but the strain contribution was also of major importance. Thus, the sensory analysis of final product gives an important insight on the overall quality.

Keywords: alcoholic fermentation; aroma profile; honey; mead; yeast immobilisation.

Introduction

Mead is a traditional honey-derived beverage containing 8 to 18 % (v/v) ethanol. The beverage is produced by yeast alcoholic fermentation of diluted honey (Mendes-Ferreira et al., 2010; Ramalhosa et al., 2011). Honey production is a significant economic activity in European countries, however to the development of honey-derived, such as mead, is of extreme importance to increase the profit of the beekeeping industry. Mead fermentation progress depends on several factors, such as yeast strain (Pereira et al., 2013), honey type and composition (Navrátil et al., 2001), lack of essential nutrients such as a deficiency in available nitrogen (Mendes-Ferreira et al., 2010), low mineral concentration, low pH (Sroka and Tuszynski, 2007) and low buffer capacity (Maugenet, 1964). Several strategies have been introduced for the optimisation of mead fermentation through the use of an appropriate honey-must formulation to improve the alcoholic fermentation performance of yeast (Mendes-Ferreira et al., 2010), using starter yeast cultures isolated from honey/honey-wine (Pereira et al., 2009; Teramoto et al., 2005) or commercial yeast starter cultures (Koguchi et al., 2009; Navrátil et al., 2001; Sroka and Tuszynski, 2007). It has been shown that supplementation of honey-must with ammonium significantly reduces fermentation length (Mendes-Ferreira et al., 2010). However, some residual sugars, other than glucose, still remain in meads after alcoholic fermentations despite the initial nitrogen concentration or the yeast strain used (Mendes-Ferreira et al., 2010). Recently, we have shown that increasing pitching rate impacts yeast fermentative activity, and significant time was saved in the fermentation process, with no detrimental impact on mead aroma composition (Pereira et al., 2013).

Microorganism immobilisation methods have gained attention in the last few decades and are being successfully applied in the production of alcohols (ethanol, butanol and isopropanol), organic acids (malic, citric, lactic and gluconic acids), enzymes (cellulose, amylase and lipase), the biotransformation of steroids for wastewater treatment and food applications (beer and wine) (Liouni et al., 2008; Reddy, et al., 2008), among others. Despite the great potential, the industrial use of immobilised cells is still limited because further application depends on the development of immobilisation procedures that can be readily scaled up (Kourkoutas et al., 2004). The main techniques that enable biomass confinement are attachment or adsorption on solid carrier surfaces, entrapment within a porous matrix, self-

aggregation of cells (flocculation) and cell containment behind a barrier (Pilkington et al., 1998). Entrapment involves imprisoning living cells within a rigid network that permits the diffusion of substrates and products, thereby making possible the growth and maintenance of active cells (Diviès and Cachon, 2005). The polymeric beads are usually spherical, with diameters ranging from 0.3 to 3 mm (Verbelen et al., 2006). Owing to the very gentle, simple and rapid procedure, the entrapment of cells in alginate hydrogels is still the most frequently used method for immobilisation (Pajic-Lijakovic et al., 2010).

The immobilised microbial cells in a hydrogel matrix are protected from harsh environmental conditions such as pH, temperature, organic solvent and inhibitors (Kocher et al., 2006; Park and Chang, 2000). Cell growth in the porous matrix depends on diffusion limitations imposed by the porosity of the material and later by the impact of accumulating biomass (Kourkoutas et al., 2004). Cell immobilisation also allows easier handling of the cells and facilitates the clarification of the final product (Kocher et al., 2006; Kostov et al., 2010; Kourkoutas et al., 2004; Park and Chang, 2000). Studies with immobilised cells in Ca-alginate (Qureshi and Tamhane, 1986) or pectate (Navrátil et al., 2001) in mead production have showed that fermentation length was reduced or fermentation rate increased, respectively. Therefore, the purpose of this study was to evaluate the effect of the yeast cell immobilisation of two yeast strains (QA23 and ICV D47) in a fed-batch system. The fermentation profile, cell viability, mead composition and mead aroma profile were evaluated in meads fermented with free or immobilised cells. Yeast cell immobilisation was accomplished using alginate high molecular hydrophilic polymeric gel at a concentration of 4%. In addition, single (Ca-alginate) or double layers (alginate-chitosan) were tested. The cells were entrapped in the gel using a drop-forming procedure.

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

A dark multifloral honey was used that was derived primarily from the pollen of *Castanea* spp. and *Erica* spp. and was purchased from a local beekeeper in the northeastern region of Portugal. The characteristics and satisfactory quality of the honey were assured in accordance with the requirements established in Portuguese law (Decreto-Lei nº 214/2003, 18th September).

Preparation of honey-must for fermentation

The honey-must for fermentation with free or immobilised cells was prepared as described by Pereira et al. (2013). The honey was diluted in natural commercially obtained spring water purchased in the market (37% w/v) to achieve 23 ºBrix, corresponding to an alcoholic beverage with approximately 13.5% ethanol and mixed to homogeneity. Then, the insoluble materials were removed from the mixture by centrifugation (2682×*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, USA), and pH was adjusted to 3.7 with malic acid (Merck, Darmstadt, Germany). The nitrogen content was adjusted to 267 mg/L with diammonium phosphate (DAP, BDH Prolabo, Leuven, Belgium). The parameters ºBrix (Optic Ivymen 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 (Organisation Internationale de la Vigne et du Vin, 2006). Yeast assimilable nitrogen (YAN) was determined by the formaldehyde method as previously described (Aerny, 1996). After clarification, 10 mL of the sample was transferred into a 50-mL beaker and diluted with 15 mL of water. The pH was adjusted to 8.1 with 100 mM NaOH (Merck, Darmstadt, Germany) and 2.5 mL of formaldehyde (Merck, Darmstadt, Germany) at pH 8.1 was added. After 5 min, the pH was adjusted again to 8.1 by titration with 50 mM NaOH. Assimilable nitrogen was calculated using the following formula:

$$\text{YAN (mg/L)} = [(\text{vol. NaOH}) \times (\text{conc. NaOH}) \times 14 \times 1000]/ (\text{sample volume})$$

The honey-must was pasteurised at 65 ºC for 10 min and then immediately cooled. No sulphur dioxide was added to the honey-must.

Immobilisation of yeast cells

Starter cultures were prepared by the rehydration of 2 g of active dry yeast in 20 mL of sterilised water at 38 ºC, according to the manufacturer's instructions, to obtain ca. 10^8 CFUs/mL. Sodium alginate (BDH Prolabo, Leuven, Belgium) was dissolved in distilled water at concentrations of 4% (w/v) and sterilised by autoclaving at 121 ºC for 20 min.

To inoculate the honey-must with 10^6 CFUs/mL, the appropriate amount of yeast suspension was added to 10 mL of sodium alginate solution. The polymer–cell mixture was added dropwise to a 180 mM $CaCl_2$ (Panreac, Barcelona, Spain) sterilised solution and left to harden in this solution for 30 min at 4 ºC. Single-layer *S. cerevisiae* immobilised beads were rinsed three times with sterile distilled water. Then, the immobilised beads were transferred into the honey-must.

For double-layer immobilisation, after the cells were left to harden in $CaCl_2$ solution for 30 min at 4 ºC, the beads were decanted and added to a chitosan (Sigma-Aldrich, St. Louis, USA) solution prepared according to Liouni et al. (2008) and maintained at 25 °C for 24 h at a rotational speed of 80 min^{-1}. Double-layer *S. cerevisiae* immobilised beads were decanted, rinsed three times with autoclaved distilled water and transferred into the honey-must.

Fermentation conditions and monitoring

The immobilised beads in the single and double layers were transferred into the honey-must for fed-batch fermentations. In parallel, free-cell fermentations with *S. cerevisiae* strain QA23 or ICV D47 were performed with 10^6 CFUs/mL for comparison with immobilised systems. All fermentations were conducted in triplicate using a previously described system (Mendes-Ferreira et al., 2010) 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), which mimicked the real industrial environment. Aseptic sampling for monitoring fermentation was performed using a syringe-type system as previously described (Mendes-Ferreira et al., 2009). The weight losses of the fermentations were monitored daily as an estimate of CO_2 production. To determine the growth parameters in the free system and of the suspended cells in the medium, 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 their CFUs in solid yeast peptone dextrose agar (YPD – 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. Determinations of reducing sugars, prior to inoculation and during fermentation, were performed using the 3,5-dinitrosalicylic acid (DNS) method with glucose as the standard. At the end of alcoholic fermentation, samples were taken from all fermented media for several analytical and aroma profile determinations.

Analyses performed at the end of fermentation

Free and immobilised cell concentrations were determined as cell dry weight. The culture dry weight of the suspended cells in the medium (from the free and immobilised systems) was determined from triplicate samples of 14 mL that were centrifuged in pre-weighed tubes at 3890.1×*g* for 10 min, washed twice with sterile deionised water, dried for 24 h at 100 °C and stored in a desiccator before weighing.

For determination of dry weight, immobilisation yield and concentration of viable cells immobilised in single and double layers, the beads were liquefied using a chemical method, according a procedure adapted from Göksungur and Zorlu (2001). Fifty beads were washed with water and dissolved in 50 mL of a 50 mM sterilised sodium citrate (Merck, Darmstadt, Germany) solution with continuous stirring for 30 min at room temperature. The dry weight of immobilised cells was determined by the same procedure described previously for the free cell system. For assessing the growth of immobilised cells, after appropriate dilutions of liquefied beads, the number of CFUs in solid YPD plates was counted after incubation at 25 °C for 48 h. The immobilisation yield was calculated as the immobilised dry weight of yeasts/immobilised and free dry weight of yeasts × 100 (Inal and Yiğitoğlu, 2011).

The oenological parameters, such as total sulphur dioxide (SO_2), pH, titratable acidity, volatile acidity and ethanol content, were determined according to standard methods (Organisation Internationale de la Vigne et du Vin, 2006). Yeast assimilable nitrogen (YAN) was determined by the formaldehyde method as previously described (Aerny, 1996).

Determination of glucose, fructose, glycerol, acetic acid and ethanol

Glucose, fructose, ethanol, glycerol and acetic acid were individually analysed, using a Varian high performance liquid chromatography (HPLC) system, equipped with a Rheodyne injector with a 20-µL loop, a Supelco Gel C—610 H column (300 mm × 7.8 mm) at 35 ºC and a refractive index detector RI -4 (Varian). Isocratic elution was employed with a mobile phase consisting of 0.1 % (v/v) phosphoric acid (Panreac, Barcelona, Spain) at a flow rate of 0.5 mL/min. Data were recorded and integrated using Star Chromatography Workstation

software (Varian). Glucose, fructose, ethanol, glycerol and acetic acid were quantified by external standard calibration.

Analysis of mead aromatic compounds

Mead produced with different immobilisation systems and the free cell system 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 achieved 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 achieved 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 ionisation 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, then programmed to rise from 60 °C to 220 °C at 3 °C min^{-1} and finally 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 of the detector response factor for each analyte, was performed with Star–Chromatography Workstation software, 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. (2006). In a 10-mL culture tube (Pyrex, ref. 1636/26MP), 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 mm × 4.8 mm) were added. 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 ionisation 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 °C to 234 °C at a rate of 3 ºC/min, raised from 234 °C to 250 ° C at 10 °C/min and finally maintained at 250 ºC 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 MS WorkStation version 6.6 software (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.

Determination of odour activity values

The Odour Activity Values (OAVs) were determined to evaluate the contribution of a certain chemical compound to the aroma of mead. Only the compounds with an OAV greater than 1 were considered to give a significant contribution to the mead's aroma (Escudero et al., 2004; Vilanova et al., 2009). The OAV was calculated for each quantified volatile compound as the ratio between the concentration of an individual compound and the perception threshold found in the literature (Escudero et al., 2004; Ferreira et al., 2000; Guth, 1997; Moreno et al., 2005).

Statistical analysis

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, were 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 yeast immobilisation on the physicochemical characteristics and aromatic compounds 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

Traditional fermentations conducted with free cells were compared with the fermentations using immobilised cells, in single- and double-layer beads. The cell concentrations in both systems were 10^6 CFUS/mL of honey-must.

Effect of immobilisation on fermentation performance

Based on previous studies performed by our group, subsequent studies were conducted with 4% Ca-alginate beads of the two yeast strains (QA23 and ICV D47). To overcome the phenomenon of cell leakage, beside the single-layer immobilisation, immobilised cells in double-layer alginate-chitosan beads were used. Fermentations with free cells were conducted in parallel with immobilised cell fermentations for comparison.

The fermentation kinetics profiles of the free or immobilised cells expressed in terms of sugar consumption are presented in Figure 5.1. In all fermentations 50 % of the sugars, or more, were consumed after 48 h of fermentation, which corresponds to an ethanol concentration of 5 to 6% vol. (data not shown). Nevertheless, fermentations conducted with different systems reached the same final ethanol concentration, 10 - 11% vol. (Table 5.2). It has been reported higher productivity in the immobilised system than in the free cell system (Nigam, 2000; Yu et al., 2007). However, it is important to state that the fermentation productivity depends on the concentration of yeast cells immobilised in beads (Inal and Yiğitoğlu, 2011), on the bead-size, as well as on the temperature of fermentation (Diviès and Cachon, 2005).

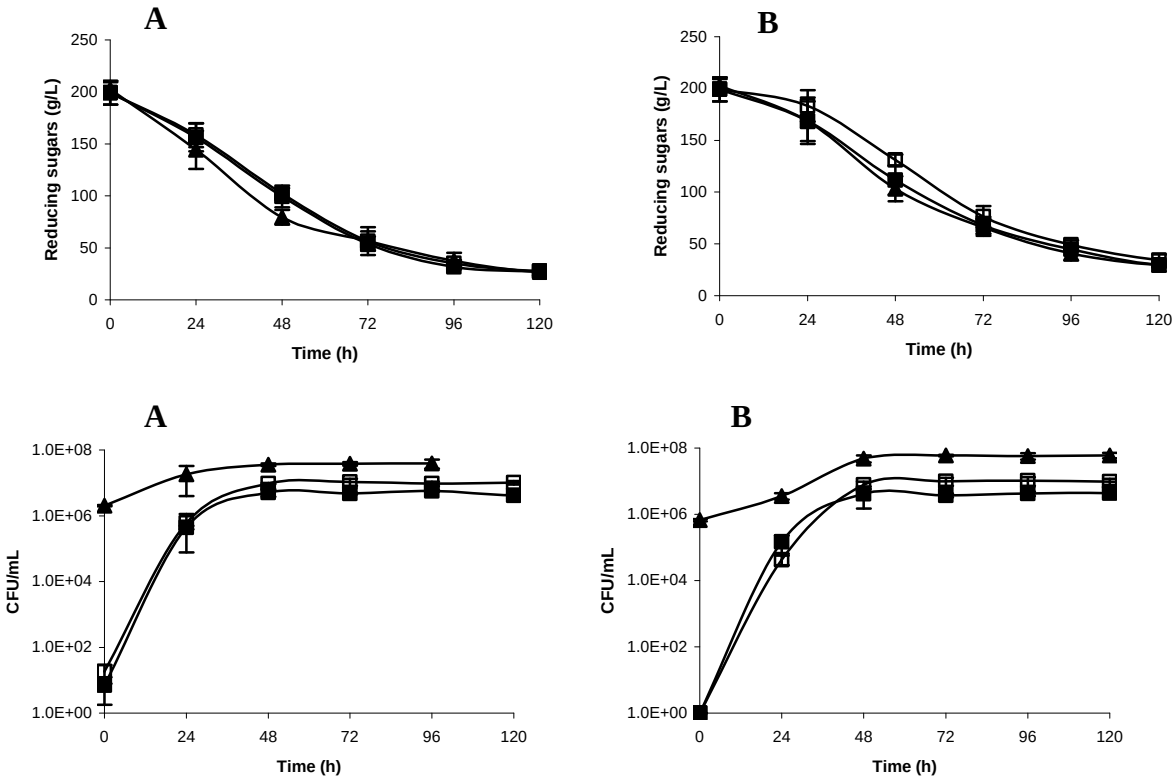

Figure 5.1. Fermentation profiles and growth kinetics of *S. cerevisiae* QA23 (A) and ICV D47 (B) cells in medium, in fermentations with free cells (▲) and immobilised cells in single- (□) or double-layer (■) beads.

Independently of the cell system, the strain ICV D47 presented lower sugar consumption in the first 48 h than the strain QA23, but fermentations performed with both strains ended 120 h after inoculation. These results are not in agreement with the findings from other studies, which observed longer fermentation length in free cells on orange peels, when compared with immobilised cells (Plessas et al., 2007). At the end of all fermentations, approximately 30 g/L of non-fermentable sugars such as trehalose, isomaltose, saccharose and melezitose (data not shown) remained in all meads, which is in agreement with results previously published by our group (Mendes-Ferreira et al., 2010; Pereira et al., 2013).

For both strains, the reducing sugar consumption profile showed a slight difference between the three cell conditions from the 24 until the 72 h of fermentation. Nevertheless, minor differences were detected between fermentations conducted with free or encapsulated cells and between the fermentations with cells immobilized in single or double-layer. In fact, Mariam et al. (2009) have already mentioned that *S. cerevisiae* consumed practically the same amount of sugar in free or in immobilised form. Regarding the efficiency of sugar consumption by encapsulated cells, Yu et al. (2007) verified that immobilized ones consumed the sugars faster and more efficiently than the free cells, while Nikolić et al. (2009) found the

opposite. The discrepancies might be due to differences in yeast strains, immobilisation agents, must composition and fermentation conditions.

Effect of immobilisation on yeast growth

Colony-forming unit (CFU) in medium analysis indicated an increase in the yeast cell population of both strains QA23 and ICV D47 within the first 24 and 48 h (Figure 5.1). The cell viability remained constant until the end of the experiments, with the number of CFUs slightly higher in fermentation with free cells, which reached almost 10^8 CFUs/mL. At the beginning of fermentations with single-layer Ca-alginate and double-layer alginate-chitosan beads no free cells were detected in the fermentation medium. However, 24 or 48 h after the onset of fermentation, depending on the strain, a considerable increase in cell population was observed, reaching 10^7 CFUs/mL. That increase in CFUs resulted from the combined effects of the cell leakage from beads, most likely due to the intensive cell growth on peripheral beads section, and to cell proliferation in the medium. Cell leakage was especially prominent during intensive carbon dioxide evolution, most likely due to the creation of pores in the polymer matrix by arising bubbles (Bezbradica et al., 2007).

It is important to note that the number of CFUs in fermentations with encapsulated cells was significantly lower than that of control fermentation reflecting the early arrest of yeast cell division most likely because the honey-must lack sufficient nutrients to support both free and encapsulated cells growth. Another explanation for the early arrest of yeast cell division under those conditions might be the space limitation due to the presence of beads. This is in agreement with the fact that no differences were detected in the number of free cells in medium fermented with single-layer Ca-alginate or double-layer alginate-chitosan beads. Due to cell leakage, it is difficult to determine the contribution of entrapped cells on the fermentation progress. To actually compare traditional fermentations with free cells with the fermentations using encapsulated cells, the cells liberated from the beads have to be periodically withdrawn from the medium.

Cell viability in beads was measured as the power of reproduction after their dissolution in sodium citrate (Table 5.1). In addition, the dry weight of cells in medium and in beads was determined (Figure 5.2). For both strains and immobilisation systems the number of CFUs in beads was higher than the number of CFUs of free cells in the outside medium, probably because encapsulated cells are more protected from such harsh environmental conditions.

Table 5.1. Total beads wet weight, CFUs and immobilisation yield of *S. cerevisiae* QA23 and ICV D47 immobilised cells in single- or double-layer beads.

	Strain QA23		**Strain ICV D47**	
	Single layer immobilisation	**Double layer immobilisation**	**Single layer immobilisation**	**Double layer immobilisation**
Total beads wet weight (g)	10.27 ± 0.27	10.73 ± 0.23	11.22 ± 0.28	11.19 ± 0.50
CFUs/mL of alginate	$2.28 \pm 0.08 \times 10^8$	$2.45 \pm 0.98 \times 10^8$	$4.87 \pm 1.51 \times 10^8$	$5.67 \pm 1.95 \times 10^8$
Immobilisation yield (%)	59.41 ± 6.30	66.79 ± 4.21	61.37 ± 6.69*	72.89 ± 2.55*

* significant at $p < 0.05$. Lack of a superscript indicates no significant difference, $p > 0.05$.

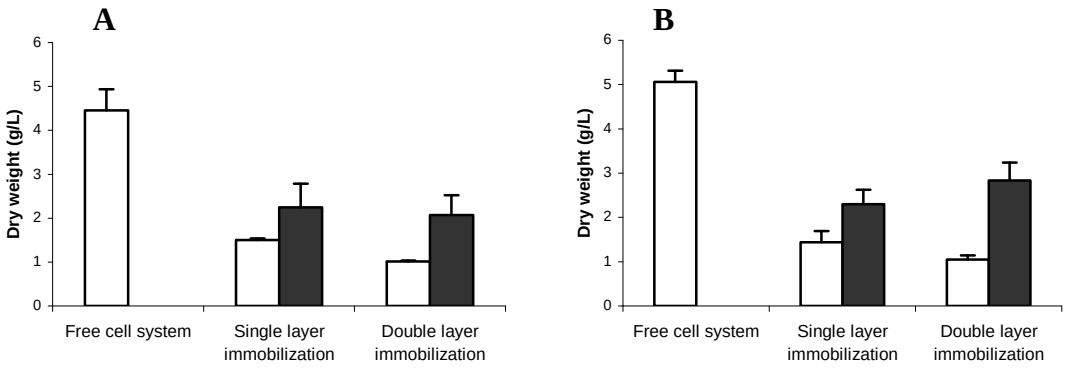

Figure 5.2. Dry weight, at the end of fermentation, of *S. cerevisiae* QA23 (A) and ICV D47 (B) cells suspended in medium (□) and in beads (■), in fermentations with free cells and immobilised cells in single- or double-layer beads.

Identical results were obtained for cell dry weight, corroborating identical observations with yeast cells immobilised in PVA particles and on orange peels (Bezbradica et al., 2007; Plessas et al., 2007). In addition, the final overall viable cell concentration in beads plus free cells in medium was higher than the total cell concentration achieved in free cell fermentation, most likely due to the high growth rate of entrapped cells in beads. Nevertheless, the final amounts of viable cells were higher in immobilised systems than in the free system, although the cellular dry weights were lower. This result indicates that the immobilisation has a negligible effect on cell viability, in agreement with previous results obtained with entrapped cells in PVA particles for beer fermentation (Bezbradica et al., 2007). The results also indicated that there was no advantage of using double-layer alginate-chitosan beads, since the final concentration of cells in medium and beads was similar. Nevertheless, an exception was

observed in the immobilisation yield of strain ICV D47, which was significant higher in double-layer fermentation.

Cell leakage is considered one of the main problems of the cell immobilisation and was especially prominent during intensive carbon dioxide evolution, most likely due to the creation of pores in the polymer matrix by arising bubbles (Bezbradica et al., 2007). Other reason for this phenomenon is the presence in the fermentation medium of high concentrations of chelating agents, such as K^+ ions and phosphate, which destroy the formatted gel matrix (Tataridis et al., 2005). Cell leakage should be minimised by double-layer immobilisation because the Ca-alginate beads were coated with chitosan. Thus, cell leakage is reduced to a significant level with the contribution of both the presence of an outer layer containing no cells coating the single-layer beads and a polyelectrolyte complex of alginate and chitosan (Liouni et al., 2008). Moreover, it is important to take into account in the immobilisation procedure the bead size to minor the phenomenon of cell leakage. Cells entrapped in small diameter beads are generally preferred because provide high solid-liquid interfacial areas per unit reactor volume and minimises mass-transfer limitation problems (Nigam, 2000; Nikolić et al., 2009). In contrast, cells immobilised in a large-size bead proliferate only in the periphery of the bead due to substrate and oxygen limitation (Park and Chang, 2000). Additionally, pH is a key factor in avoiding cell leakage because it affects the mechanical stability and integrity of the beads (Vilela et al., 2013).

Effect of immobilisation on mead quality

The physicochemical characteristics such as pH, volatile acidity, titratable acidity, final assimilable nitrogen, total SO_2, ethanol and reducing sugars of meads produced by strain QA23 and ICV D47 with free cells and different immobilisation systems are presented in Table 5.2. The final pH of meads was lower than the initial pH of honey-must but no significant differences were found between cell conditions for both strains. Identical observations were verified for titratable acidity and total SO_2.

Table 5.2. Physicochemical characteristics of honey-must and meads fermented by *S. cerevisiae* QA23 and ICV D47 with free cells and immobilised cells in single- or double-layer beads. The results are shown as the mean values and their standard deviations.

Honey-musts	**Prior adjustment**	**After adjustment**
pH	4.54 ± 0.05	3.71 ± 0.01
º Brix (%)	22.60 ± 0.40	23.30 ± 0.20
Titratable acidity $_{\text{tartaric acid}}$ (g/L)	0.79 ± 0.17	4.94 ± 0.84
Initial nitrogen $_{\text{YAN}}$ (mg/L)	48.30 ± 5.75	273.00 ± 22.55

	QA 23			**ICV D47**		
Meads	**Free system**	**Single layer immobilisation**	**Double layer immobilisation**	**Free system**	**Single layer immobilisation**	**Double layer immobilisation**
pH	3.67 ± 0.05	3.62 ± 0.10	3.63 ± 0.12	3.60 ± 0.06	3.60 ± 0.08	3.62 ± 0.07
Volatile acidity $_{\text{acetic acid}}$ (g/L)	0.43 ± 0.02	0.50 ± 0.03	0.50 ± 0.05	0.34 ± 0.03 ^{a}	0.56 ± 0.03 ^{b}	0.58 ± 0.03 ^{b}
Titratable acidity $_{\text{tartaric acid}}$ (g/L)	6.58 ± 0.27	8.75 ± 2.31	8.56 ± 2.15	6.96 ± 0.15	8.94 ± 2.23	9.04 ± 2.34
Final nitrogen $_{\text{YAN}}$ (mg/L)	33.83 ± 2.02	31.50 ± 3.50	26.25 ± 5.25	33.83 ± 4.04 ^{b}	23.33 ± 4.04 ^{a}	31.50 ± 3.50 ^{ab}
Total SO_2 (mg/L)	25.60 ± 2.56	23.89 ± 3.91	21.76 ± 1.28	26.03 ± 3.22	23.47 ± 3.70	23.04 ± 2.56
Ethanol (% vol)	11.20 ± 0.00 ^{b}	10.53 ± 0.12 ^{a}	10.81 ± 0.32 ^{ab}	10.87 ± 0.12	10.73 ± 0.32	10.73 ± 0.20

$^{a–b}$ Means within a line with different superscripts differ, $p < 0.05$. Lack of a superscript indicates no significant difference, $p > 0.05$.

Volatile acidity, expressed as g/L of acetic acid, confirms the values of acetic acid obtained by HPLC (Figure 5.3) and showed differences between free and immobilised fermentations with strain ICV D47. The ethanol concentration ranged from 10.53 to 11.20% vol. in meads produced by the strain QA23 with immobilised cells in single-layer Ca-alginate or free cells, respectively. A similar concentration of residual assimilable nitrogen remained in all meads independently of the strain and the condition of cells, most likely corresponding to the concentration of the amino acid proline, which is present in honey but not assimilable by yeasts (Pereira et al., 2013). Concerning the strain ICV D47, the consumption of nitrogen by immobilised cells in single-layer Ca-alginate was significant higher than the consumption by free cells. These results are in accordance with high cell growth in immobilised systems due to the growth of cells inside the beads and in the medium, which can explain the higher consumption of nitrogen in the immobilised system. Others have reported a low consumption of free amino nitrogen linked to a very limited or no cell growth in immobilised yeast systems (Willaert and Nedovic, 2006).

The concentration of glycerol produced by both strains at the end of fermentations ranged from 5.3 to 6.6 g/L (Figure 5.3). For both strains, at the end of fermentations, a significant difference was found (results not shown) in glycerol concentration produced by free cells or cells immobilised in double-layer. Higher concentration of glycerol was obtained for the fermentations conducted with free cells. The concentrations of this alcohol determined in all assays were in agreement with the values usually reported in wine (Ribéreau-Gayon et al., 2000; Ugliano and Henschke, 2009) and in meads (Pereira et al., 2009). Environmental factors such as temperature, aeration, nitrogen source, sugar concentration and the yeast strain have been found to affect the rate and yield of glycerol production (Remize et al. 2000). A significant increase in glycerol formation by immobilised cells of *S. cerevisiae* has been reported in the production of alcohol-free beer (van Iersel et al. 2000), of wine (Balli et al. 2003; Reddy et al. 2011) as well as in other fruit-fermented beverage (Oliveira et al. 2011). In contrast, other authors have observed higher amounts of glycerol in fermentations performed with free cells (Genisheva et al. 2012; López de Lerma et al. 2012). High glycerol production in fermented products using immobilised cells may be a yeast response to the stress conditions imposed by this system (Reddy et al. 2011). Nevertheless, no explanation has been proposed before for the increase in glycerol production by free cells.

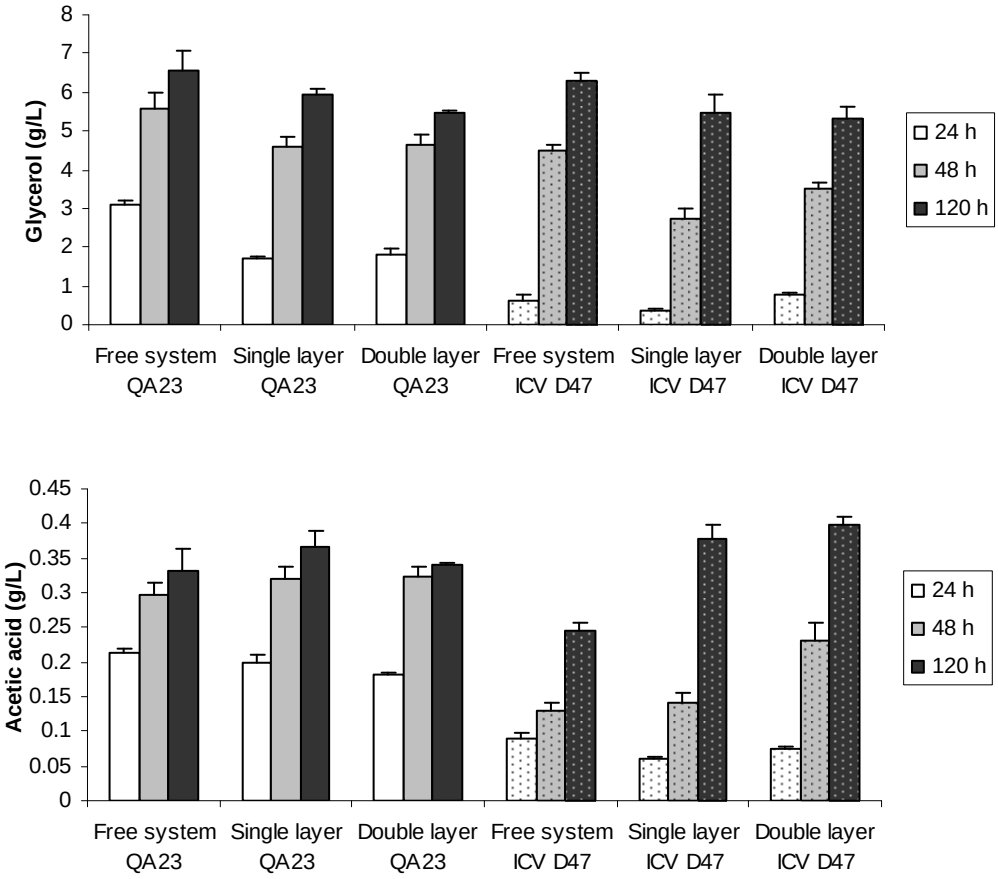

Figure 5.3. Concentration of glycerol and acetic acid produced by *S. cerevisiae* QA23 and ICV D47 after 24, 48 and 120 h of fermentation with free cells and immobilised cells in single- or double-layer beads.

In respect to acetic acid production, the immobilisation process had a distinct effect on strain QA23 and ICV D47 (Figure 5.3). For strain QA23, the immobilisation did not affect the acetic acid production in mead, where the concentration was approximately 0.3 g/L in all meads. Instead, the strain ICV D47 produced almost the double of acetic acid in immobilised than in the free form. Indeed, the difference in acetic acid production between strains or cell conditions has already been reported (Genisheva et al., 2012; van Iersel et al., 2000; López de Lerma et al., 2012; Oliveira et al., 2011). The discrepancies among the results obtained may be explained by differences in yeast strains, medium composition and fermentation conditions. In fact, as previously stated, meads obtained with strain ICV D47 displayed lower volatile acidity than the meads produced by strain QA23, both in the free form (Pereira et al., 2013). Similar concentrations have been observed in mead produced from Portuguese honey (Pereira et al., 2009), whereas Sroka and Tuszyński (2007) reported higher concentrations (0.75 g/L).

Effect of immobilisation on mead aroma profile

The alcoholic fermentation produces not only ethanol but also a complex mixture of flavour-active by-products. The concentrations of volatile compounds in meads produced by strain QA23 and ICV D47 immobilised in single-layer Ca-alginate or double-layer alginate-chitosan and in free form are shown in Tables 5.3 and 5.4, respectively. A total of twenty-five compounds were identified and quantified, including alcohols, esters, volatile phenols and medium chain fatty acids.

The alcohols were the major group of volatile compounds quantified in all meads. No correlation could be established between the concentration of alcohols and the condition of cells (immobilised or free). The strain QA23 produced less alcohol in meads fermented with single-layer Ca-alginate immobilised cells, whereas the strain ICV D47 produced the lowest concentration of this group of compounds in meads fermented with cells immobilised in double-layer alginate-chitosan (data not shown). However, as desirable for the complexity of alcoholic beverage the concentration of these alcohols should be below 300 mg/L (Boulton et al., 1996; Ugliano and Henschke, 2009). The major alcohol detected in all meads was 3-methyl-1-butanol at a concentration above its perception threshold. In general, the concentration of alcohol compounds decreased or was similar in meads obtained with immobilised cells, with the exception of 1-propanol. That alcohol was more produced by immobilised cells over a range of 20.16 - 31.62 mg/L for strain QA23 and 24.12 - 60.12 mg/L for strain ICV D47. Identical values of 1-propanol to the ones obtained in fermentation with QA23 immobilised in single-layer Ca-alginate, were observed by Plessas et al. (2007) in anaerobic batch fermentations of glucose by *S. cerevisiae* cells immobilised on orange peels. Normally, the significant lower amounts of 2-methyl-1-butanol and 2-phenylethanol were produced by cells immobilised. Higher alcohols individually do not give pleasant notes to the beverage, except 2-phenylethanol, but together they can contribute positively to the overall aroma (Genisheva et al., 2012). The concentration of 2-phenylethanol in all meads was above their perception threshold, 14 mg/L (Escudero et al., 2004, Guth, 1997), and similar concentrations have already been reported in wines fermented with free or immobilised cells of *S. cerevisiae* (Genisheva et al., 2012).

Table 5.3. Concentration of volatile compounds of meads fermented *S. cerevisiae* QA23 with free cells and immobilised cells in single- or double-layer beads. The results are shown as the mean values and their standard deviations.

	Free cell system	Single-layer immobilisation	Double-layer immobilisation
Alcohols (mg/L)			
1-propanol	20.16 ± 1.05 [a]	25.45 ± 6.02 [ab]	31.62 ± 3.47 [b]
2-methyl-1-propanol	23.98 ± 2.29 [b]	17.33 ± 1.97 [a]	20.76 ± 1.56 [ab]
2-methyl-1-butanol	18.80 ± 2.73 [b]	13.28 ± 1.70 [a]	16.09 ± 1.74 [ab]
3-methyl-1-butanol	141.86 ± 18.93	120.59 ± 19.48	143.49 ± 16.54
2-phenylethanol	29.09 ± 3.43 [b]	21.06 ± 1.91 [a]	27.52 ± 1.93 [b]
3-ethoxy-1-propanol	0.13 ± 0.04	0.16 ± 0.08	0.16 ± 0.05
3-(methylthio)-1-propanol	0.07 ± 0.02 [b]	0.04 ± 0.01 [ab]	0.04 ± 0.004 [a]
Esters (mg/L)			
ethyl acetate	35.66 ± 3.15	44.54 ± 10.10	53.46 ± 10.42
ethyl butyrate	0.10 ± 0.01	0.12 ± 0.03	0.12 ± 0.01
isoamyl acetate	1.15 ± 0.05	1.28 ± 0.38	1.27 ± 0.20
ethyl hexanoate	0.21 ± 0.02	0.25 ± 0.05	0.27 ± 0.03
ethyl lactate	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
ethyl octanoate	0.14 ± 0.04 [a]	0.23 ± 0.03 [ab]	0.25 ± 0.06 [b]
ethyl decanoate	0.06 ± 0.03 [a]	0.12 ± 0.02 [ab]	0.17 ± 0.05 [b]
ethyl phenylacetate	0.002 ± 0.001	0.002 ± 0.000	0.002 ± 0.001
2-phenylethyl acetate	0.46 ± 0.12	0.52 ± 0.14	0.41 ± 0.05
Volatile phenols (µg/L)			
4-vinylguaiacol	79.17 ± 17.90	75.99 ± 14.40	80.23 ± 7.53
4-vinylphenol	115.06 ± 21.10	111.53 ± 26.53	112.86 ± 14.88
Medium chain fatty acids (µg/L)			
isobutyric acid	25.59 ± 5.54 [b]	12.34 ± 4.24 [a]	10.68 ± 1.90 [a]
butanoic acid	10.96 ± 3.82	10.00 ± 4.04	8.69 ± 2.05
hexanoic acid	510.42 ± 141.89	557.86 ± 158.16	527.63 ± 81.50
octanoic acid	1533.17 ± 287.61	1880.71 ± 456.45	1934.90 ± 175.73
decanoic acid	268.94 ± 60.59	358.03 ± 149.45	469.90 ± 30.82
dodecanoic acid	5.10 ± 4.08	3.31 ± 1.18	1.84 ± 0.82
Carbonyl compounds (mg/L)			
acetaldehyde	7.45 ± 1.60	5.02 ± 0.46	8.27 ± 3.38

[a–b] Means within a line with different superscripts differ, $p < 0.05$. Lack of a superscript indicates no significant difference, $p > 0.05$.

Table 5.4. Concentration of volatile compounds of meads fermented *S. cerevisiae* ICV D47 with free cells and immobilised cells in single- or double-layer beads. The results are shown as the mean values and their standard deviations.

	Free cell system	Single-layer immobilisation	Double-layer immobilisation
Alcohols (mg/L)			
1-propanol	24.12 ± 1.32 [a]	60.12 ± 3.20 [b]	57.97 ± 10.64 [b]
2-methyl-1-propanol	20.88 ± 1.30	22.04 ± 1.02	19.91 ± 0.33
2-methyl-1-butanol	22.15 ± 1.50 [b]	18.27 ± 1.66 [a]	15.19 ± 1.02 [a]
3-methyl-1-butanol	157.26 ± 6.87 [b]	141.20 ± 13.98 [ab]	120.03 ± 6.45 [a]
2-phenylethanol	33.68 ± 2.35 [b]	33.89 ± 4.89 [b]	17.96 ± 1.10 [a]
3-ethoxy-1-propanol	0.01 ± 0.003	0.01 ± 0.003	0.01 ± 0.001
3-(methylthio)-1-propanol	0.08 ± 0.01 [b]	0.02 ± 0.005 [a]	0.02 ± 0.01 [a]
Esters (mg/L)			
ethyl acetate	35.41 ± 4.15 [a]	49.92 ± 1.11 [b]	50.75 ± 1.50 [b]
ethyl butyrate	0.09 ± 0.01 [a]	0.11 ± 0.01 [ab]	0.12 ± 0.01 [b]
isoamyl acetate	1.61 ± 0.28 [b]	1.06 ± 0.05 [a]	1.16 ± 0.12 [ab]
ethyl hexanoate	0.18 ± 0.01 [a]	0.26 ± 0.02 [b]	0.27 ± 0.04 [b]
ethyl lactate	0.03 ± 0.004	0.03 ± 0.01	0.03 ± 0.003
ethyl octanoate	0.10 ± 0.01	0.16 ± 0.04	0.17 ± 0.03
ethyl decanoate	0.04 ± 0.01	0.06 ± 0.02	0.05 ± 0.005
ethyl phenylacetate	0.002 ± 0.000	0.001 ± 0.000	0.002 ± 0.000
2-phenylethyl acetate	0.70 ± 0.05 [b]	0.28 ± 0.02 [a]	0.24 ± 0.04 [a]
Volatile phenols (µg/L)			
4-vinylguaiacol	91.55 ± 12.24	82.82 ± 4.34	83.85 ± 20.06
4-vinylphenol	118.78 ± 21.53	104.73 ± 7.25	102.72 ± 27.13
Medium chain fatty acids (µg/L)			
isobutyric acid	23.02 ± 5.95 [b]	11.11 ± 2.71 [a]	10.53 ± 2.88 [a]
butanoic acid	8.67 ± 3.61	9.77 ± 3.29	10.23 ± 2.68
hexanoic acid	426.20 ± 90.90	508.39 ± 95.45	504.31 ± 78.59
octanoic acid	1439.98 ± 71.23 [a]	1557.63 ± 166.10 [ab]	1799.55 ± 153.28 [b]
decanoic acid	224.37 ± 24.09	205.02 ± 45.85	294.28 ± 35.27
dodecanoic acid	2.10 ± 0.81	1.84 ± 0.83	1.05 ± 0.43
Carbonyl compounds (mg/L)			
acetaldehyde	5.87 ± 0.33	11.43 ± 4.68	6.14 ± 1.26

[a–b] Means within a line with different superscripts differ, $p < 0.05$. Lack of a superscript indicates no significant difference, $p > 0.05$.

The second major group of compounds quantified in meads was the esters, which give fruity/flowery nuances to the aroma of fermented beverages (Swiegers et al., 2005; Willaert and Nedovic, 2006). Meads obtained with immobilised cells presented higher concentrations of esters, but no remarkable differences were detected between the two strains, as reported in literature (Willaert and Nedovic, 2006). The major ester in all meads was ethyl acetate, with a concentration ranging from 35.41 to 53.46 mg/L, in accordance to previous results on mead fermentation (Mendes-Ferreira et al., 2010). Larger amounts of ethyl acetate were produced by cells immobilised in double-layer alginate-chitosan, and the strain IVC D47 produced significant less ethyl acetate, when free cell systems were applied. These results are in accordance to Genisheva et al. (2012) and Reddy et al. (2011) who observed higher concentrations of ethyl acetate in fermentations using immobilised cells, whereas Tsakiris et al. (2004) observed higher concentrations in fermentations using free cells. The strain QA23 produced ethyl octanoate and ethyl decanoate in significant higher amounts immobilised in double-layer alginate-chitosan than in the free form. Different results were obtained in fermentation with the strain ICV D47, which produced more 2-phenylethyl acetate using free cells and oppositely produced more ethyl hexanoate in fermentations using single- or double-layer cells. In contrast, Genisheva et al. (2012) found higher amounts of both esters in fermentations using immobilised cells.

The volatile phenols, 4-vinylphenol and 4-vinylguaiacol, considered as off-flavours because they give an unpleasant aroma of wet animal (Swiegers et al., 2005), were quantified herein at concentrations below their perception threshold. However, no significant differences were detected between the strains or cells conditions.

Six medium-chain fatty acids (MCFA) were identified and quantified in all meads. Octanoic acid was the major MCFA quantified in all meads, and it was observed in concentrations above its perception threshold (0.5 mg/L). The concentration of this compound was highly variable in fermentations using free or immobilised cells in double-layer of the strain ICV D47, whereas no differences were detected in fermentations conducted with the strain QA23. High concentrations of octanoic and decanoic acids have been reported in wines obtained with immobilised cells on grape pomace peels (Genisheva et al., 2012). Isobutyric acid was the only MCFA that displayed significant differences in its concentrations for both strains, depending on the system used (free or immobilised).

The concentration of acetaldehyde ranged from 5.02 to 11.43 mg/L, always above its perception threshold (0.5 mg/L), and no differences were found between cells conditions for

both strains. Tsakiris et al. (2004) reported high amounts of acetaldehyde in fermentations with free cells compared to fermentations using cells immobilised on dried raisin berries.

In summary, the major differences between our findings and other studies result from the use of different strains, different fermentation conditions and media composition. In fact, the differences in mead flavour are most likely determined by amino acid metabolism and thus the growth of the yeast cells (Verbelen et al., 2006).

To evaluate the contribution of the volatile compounds to the aroma of mead, the odour activity values (OAVs) were determined. However, individual OAVs can serve as estimates for the potential contribution of each compound to the global aroma, but do not account for the antagonistic or synergistic effects resulting from the perceptual interactions between different molecules present in wines (Vilanova et al., 2009). The OAVs of volatile compounds with more influence on mead aroma profile are presented in Table 5.5. Only eleven volatile compounds out of the twenty-five quantified most likely have a more significant contribution to mead's aroma. The most aromatic meads were produced by strain QA23 immobilised in double-layer alginate-chitosan. In general, the meads produced by strain ICV D47 were less aromatic than the ones obtained with strain QA23 in agreement with previous results obtained with the same strains but with different inocula size (Pereira et al., 2013). The most aromatic mead produced by strain ICV D47 was the one fermented with cells immobilised in single-layer Ca-alginate. Indeed, the less aromatic meads were the ones obtained with free cells, irrespective the yeast strain used. However, the OAVs of the undesirable compounds, such as ethyl acetate, octanoic acid and hexanoic acid, were higher in fermentations using immobilised cells. The most powerful odorants in meads were ethyl octanoate, isoamyl acetate and ethyl hexanoate, as already reported in literature (Pereira et al., 2013). All these esters, contribute with desirable characteristics, such as floral/fruity notes for mead aroma profile (Guth, 1997; Moreno et al., 2005). Interestingly, the OAVs of these three compounds were higher in fermentations using immobilised cells.

Table 5.5. Odour activity values (OAV) of volatile compounds of more influence on the aroma of meads fermented by *S. cerevisiae* QA23 and ICV D47 in fermentations with free cells and immobilised cells in single- or double-layer beads.

Compounds	Odour descriptor [a]	Odour threshold (µg/L) [a]	QA23			ICV D47		
			Free cell system	Single layer immobilisation	Double layer immobilisation	Free cell system	Single layer immobilisation	Double layer immobilisation
3-methyl-1-butanol	Cheese; nail polish	30 000	4.7	4.0	4.8	5.2	4.7	4.0
2-phenylethanol	Roses; flowery	14 000	2.1	1.5	2.0	2.4	2.4	1.3
ethyl butyrate	Fruity; sweet	20	5.0	5.8	5.9	4.5	5.7	6.0
ethyl hexanoate	Fruity; aniseed	14	15.2	18.1	19.6	13.1	18.4	19.5
ethyl octanoate	Fruity; sweet	5	27.8	46.4	50.6	19.7	32.3	33.5
ethyl acetate	Solvent; nail polish	12 300	2.9	3.6	4.3	2.9	4.1	4.1
isoamyl acetate	Banana	30	38.2	42.8	42.3	53.6	35.4	38.5
2-phenylethyl acetate	Flowery; roses	250	1.9	2.1	1.6	2.8	1.1	1.0
hexanoic acid	Cheese; sweaty	420	1.2	1.3	1.3	1.0	1.2	1.2
octanoic acid	Fatty; rancid	500	3.1	3.8	3.9	2.9	3.1	3.6
acetaldehyde	Fresh; green leaves	500	14.9	10.0	16.5	11.7	22.9	12.3

[a] Odour descriptors and odour threshold reported in the literature (Guth, 1997, Moreno *et al*, 2005, Escudero *et al*, 2004, Ferreira *et al*, 2000).

Conclusions

The present study aimed to evaluate the effect of using immobilised cell systems on mead production. Our results demonstrate that the immobilisation of yeasts in Ca-alginate did not negatively affect the fermentation process. Minor differences were detected in the fermentation length and in the rate between fermentations conducted with free or immobilised cells, even though higher concentrations of viable cells were achieved in immobilised systems. The phenomenon of cell leakage, one of the major problems encountered in cell immobilisation was not reduced by the use of double-layer alginate-chitosan, and was probably responsible for the main differences observed between free and immobilised fermentations.

Although the most aromatic meads were the ones produced by immobilised cells, the OAVs of undesirable compounds were also higher in these fermentations. It appears that immobilisation has minor advantages for mead production. Despite this, the scale-up of the process can be studied because of unrealised cost advantages, several engineering problems and altered yeast physiological and metabolic properties, which may influence the flavour of the beverage or the fermentation performance. Sensorial analysis of meads could complement the analysis of aroma compounds and therefore allow inferring about its acceptance by consumers.

CHAPTER 6

Volatile composition and sensory properties of mead

In preparation for submission

Abstract

Mead is a traditional beverage that results from the alcoholic fermentation of diluted honey performed by yeasts. Several studies have been currently performed to optimize the production of this beverage. Contrary to other alcoholic beverages, only few studies have been done on the physicochemical characterization of mead and much less it has been done on its sensory properties. So, the main objective of this work was to assess if there is or not a correlation between aroma compound formation by yeasts and the sensory attributes of mead. Thus, free and immobilised cells of two yeast strains of *Saccharomyces cerevisiae*, QA23 and ICV D47, were used for mead fermentation. The quality of mead was assessed by determination of several physicochemical characteristics, aroma compound formation and sensory analysis in final product. The results revealed that both the "strain" and the "condition" had a significant effect on final pH, volatile acidity and final concentration of fructose. Regarding mead aroma composition, a total of twenty-seven compounds were identified and quantified, including alcohols, esters, volatile phenols and medium chain fatty acids. The effect of "condition" was more important than the "strain" in the volatile composition of mead. Even so, only fourteen of the twenty-seven compounds quantified were present in some samples at concentrations higher than their corresponding odour thresholds (OAV > 1), thus contributing to mead final aroma. The OAVs showed that yeast free cells produced more pleasant volatile compounds than immobilized cells, displaying the strain QA23 more aroma potential. In respect to sensory analysis the most pleasant aroma descriptors were correlated with mead obtained with yeast free cells, independently of the strain. In short, either sensory analysis or volatile composition, indicate that the most pleasant mead was produce by free yeast cells.

Keywords: aroma volatile compounds, mead, sensory analysis

Introduction

Aroma volatile compounds play a key role in determining the quality of wines because are the primary contributors to aroma and produce an effect on the sensory senses of the taster (Andreu-Sevilla et al., 2013; Vilanova et al., 2010). Wine aroma is composed of varietal aroma that arises directly from the grapes with minor modifications; fermentation aroma, produced by yeasts during the alcoholic fermentation; and the maturation *bouquet* that results from chemical reactions during ageing (Mendes-Ferreira et al., 2011; Robinson et al., 2011; Swiegers et al., 2005; Vilanova et al., 2010). Indeed, alcoholic fermentation increases the number and total concentration of the volatiles initially present in grape must and enhances the aroma properties of the fruit leading to a characteristic, aromatic and healthy fruit wine (Andreu-Sevilla et al., 2013). The combination of volatile compounds defines the quality of the beverage and therefore allows the distinction of different beverages (Andreu-Sevilla et al., 2013).

Two main types of methods for evaluation of the quality of beverages and foods can be used, namely, subjective and objective. An example of an objective analysis technique is the identification and quantification of aroma volatile compounds by gas chromatography (GC). However to asses the contribution of an individual compound in the overall aroma it is important the determination of the odour activity value (OAV). In general, the aroma active compounds are volatiles whose concentration in beverage is above their perception threshold (OAV > 1). However, it is necessary take into account the additive or synergistic effect among different volatile compounds (Vilanova et al., 2012; Vilanova et al., 2013). Subjective methods are based on human assessment of the quality characteristics of the food (Smyth and Cozzolino, 2013). For instance, sensory analysis is indispensable for the assessment of food flavour characteristics to identify the significant sensory and quality contributors to food quality and consumer preference (Schmidtke et al., 2010). Overall, the most important sensory characteristics of beverages are evaluated through the smell, the taste and to a lesser extent, the colour and the appearance (Robinson et al., 2011) and are performed by a panel of experts or consumers. However, the flavour of a drink is a sensory perception that varies with the individual, the context of the consumer experience and the chemical composition of the product (Schmidtke et al., 2010).

The establishment of correlations between sensory characteristics and instrumental measurements of specific compositional attributes can lead to a better understanding of the

relationship between compositions and sensory properties (Schmidtke et al., 2010). The correlation between instrumental and sensory data has been explored to establish the wine quality (Andreu-Sevilla et al., 2013; Vilanova et al., 2010; Vilanova et al., 2012; Vilanova et al., 2013).

Mead is traditional alcoholic beverage, still not industrialized that results from the fermentation performed by yeasts of honey diluted in water. Although the scientific improvements on honey-must formulation, fermentation conditions and yeast performance (Chen et al., 2013; Gomes et al., 2013; Mendes-Ferreira et al., 2010; Navrátil et al., 2001; Pereira et al., 2009, 2013, 2014b and 2015; Qureshi and Tamhane, 1986; Roldán et al., 2011), there is a lack of information about sensory quality of mead. The aroma of mead has contributions from honey, yeast and processes conditions (Chen et al., 2013; Gupta and Sharma, 2009). The identification and quantification of aroma compounds in mead produced under different conditions has been assessed (Mendes-Ferreira et al., 2010; Pereira et al., 2013 and 2014b; Šmogrovičová et al., 2012; Sroka and Tuszyński, 2007). However, as far as we know, there are only two works focused on mead sensory profile. One is about the influence of pollen addition on physicochemical and sensory characteristics of mead (Roldán et al., 2011). The other was about the sensory characteristics of mead produced with cassava floral honey (Ukpabi, 2006). Indeed, there is a need of performing sensory analysis for better understanding how the volatile aroma profile may interfere on consumer's acceptance.

Therefore, the first aim of this work was to characterize the quality, including the identification and quantification of volatile compounds of mead produced by free and immobilised cells of two different yeast strains of *Saccharomyces cerevisiae*, QA23 and ICV D47. The second objective was to analyse the sensory properties of mead and correlate the volatile compounds identified with differences detected in aroma attributes of mead.

Material and Methods

Yeast strains and honey

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.

A dark multifloral honey was used that was derived primarily from the pollen of *Castanea* spp. and *Erica* spp. and was purchased from a local beekeeper in the northeastern region of Portugal. The characteristics and satisfactory quality of the honey were assured in accordance with the requirements established in Portuguese law (Decreto-Lei nº 214/2003, 18th September).

Immobilisation of yeast cells

Starter cultures were prepared by the rehydration of the active dry yeasts according to the manufacturer's instructions, to obtain 10^8 CFUs/mL. To inoculate the honey-must with 10^6 CFUs/mL, the appropriate amount of yeast suspension was added to a 4 % (w/v) sterilised sodium alginate (BDH Prolabo, Leuven, Belgium) solution. The polymer–cell mixture was added dropwise to a 180 mM $CaCl_2$ (Panreac, Barcelona, Spain) sterilised solution and left to harden in this solution for 30 min at 4 ºC. *S. cerevisiae* immobilised beads were rinsed three times with sterile distilled water. Then, the immobilised beads were transferred into the honey-must.

Honey-must and fermentation conditions

The honey-must for fermentation with free or immobilised cells was prepared as described by Pereira et al. (2013). The honey was diluted in natural spring water (37 % w/v) to obtain an alcoholic beverage with approximately 11 % ethanol. Titratable acidity was adjusted with 5 g/L of potassium tartrate (Sigma-Aldrich, St. Louis, USA), and pH was adjusted to 3.7 with malic acid (Merck, Darmstadt, Germany). The nitrogen content was adjusted to 267 mg/L with diammonium phosphate (DAP, BDH Prolabo, Leuven, Belgium). The parameters ºBrix, pH, total acidity and assimilable nitrogen concentration were determined prior to and after the adjustments. The honey-must was divided into 2 L glass vessels and inoculated with approximately 10^6 CFUs/mL of *S. cerevisiae* strain QA23 or ICV D47 in the immobilised or free form. All fermentations were conducted in duplicate. The glass vessels were maintained during alcoholic fermentation at 25 °C under permanent but moderate shaking (120 rpm), which mimicked the real industrial environment. Fermentations were monitored daily by the weight loss as an estimate of CO_2 production. At the end of alcoholic fermentation, the mead was centrifuged for further analysis (enological, aroma and sensory determinations).

General oenological parameters

The reducing sugars were determined using the 3,5-dinitrosalicylic acid (DNS) method with glucose as standard. The oenological parameters, such as total sulphur dioxide (SO_2), pH, titratable acidity, volatile acidity and ethanol content, were determined according to standard methods (Organisation Internationale de la Vigne et du Vin, 2006). Yeast assimilable nitrogen (YAN) was determined by the formaldehyde method as previously described (Aerny, 1996).

Determination of glucose, fructose, glycerol, acetic acid and ethanol

Glucose, fructose, ethanol, glycerol and acetic acid were individually analysed, using a Varian high performance liquid chromatography (HPLC) system, equipped with a Rheodyne injector with a 20-µL loop, a Supelco Gel C—610 H column (300 mm × 7.8 mm) at 35 ºC and a refractive index detector RI -4 (Varian). Isocratic elution was employed with a mobile phase consisting of 0.1 % (v/v) phosphoric acid (Panreac, Barcelona, Spain) at a flow rate of 0.5 mL/min. Data were recorded and integrated using Star Chromatography Workstation software (Varian). Glucose, fructose, ethanol, glycerol and acetic acid were quantified by external standard calibration.

Analysis of mead aromatic compounds

Mead produced with different immobilisation systems and the free cell system 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 achieved 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 achieved 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 3540 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 ionisation 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, then programmed to rise from 60 °C to 220 °C at 3 °C min^{-1} and finally 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 of the detector response factor for each analyte, was performed with Star–Chromatography Workstation software, 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. (2006). In a 10-mL culture tube (Pyrex, ref. 1636/26MP), 8 mL of mead clarified by centrifugation, 100 µL of an ethanolic solution, 35.4 mg/L of an internal standard (4-nonanol, Merck, Darmstadt, Germany) and a magnetic stir bar (22.2 mm × 4.8 mm) were added. 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 ionisation 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 °C to 234 °C at a rate of 3 ºC/min, raised from 234 °C to 250 ° C at 10 °C/min and finally maintained at 250 ºC 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 MS WorkStation version 6.6 software

(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 (Boidron et al., 1988; Escudero et al., 2004; Ferreira et al., 2000; Guth, 1997; Moreno et al., 2005).

Sensory analysis

The evaluation of mead by sensory analysis was performed using the methodology described in Standards ISO 4121 (International Organisation for Standardization, 2003) and ISO 6658 (International Organisation for Standardization, 2005). The sensory attributes evaluated were divided into 3 groups: appearance (color and turbidity), taste (sweet, sour and astringency) and aroma (fruity, honey, vegetable, alcohol and chemical). These attributes were selected by reference to those normally used in sensory analysis of semi-sweet white wines. The intensity of each parameter was measured using a 7-point scale, corresponding 1 to "missing or invalid" and 7 "very strong". Finally, the overall impression of each mead sample was evaluated using a scale of 1 to 10. All analyzes were carried out by a panel of 16 semi-trained tasters.

Statistical analysis

The chemical, HPLC and volatile data were analysed using a SPSS software, version 17.0 (SPSS, Inc.). To test significant differences among physicochemical characteristics and aromatic compounds of mead, a two factor – strain (S) and condition (C) – analysis of variance (ANOVA) was applied. In order to compare the means if a Tukey's honestly significant difference multiple comparison test was used. 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 statistical tests were performed at a 5% significance level.

The sensory data was analysed using the software XLstat program with Excel from Microsoft Office, following the internet tutorial from XLSTAT (PrefMap) (2006).

Principal Component Analysis (PCA) is one of the most commonly used multivariate techniques for grape and wine analysis (Oliveira et al., 2011; Tao et al., 2009; Vilanova et al.,

2010). For interpreting the results PCA on volatile compounds (OAV > 1) and aroma descriptors was applied. PCA provides a very simple method for characterizing multidimensional data and it was used to relate the volatile compounds with OAV > 1 and the different aroma attributes with mead.

Results and Discussion

General physicochemical characterization of mead

The values of the classical physicochemical parameters of mead produced by the strains QA23 and ICV D47, under free or immobilised form, are displayed in Table 6.1.

Table 6.1. Physicochemical characteristics of mead fermented by *S. cerevisiae* QA23 and ICV D47 with free cells (F) or immobilised cells (I) and significance of the factors strain (S) and condition (C) according to two-way ANOVA.

	QA23 F	QA23 I	ICV D47 F	ICV D47 I	Significance		
					Strain	Condition	S x C
pH	3.48 ± 0.01^{ab}	3.53 ± 0.01^{b}	3.46 ± 0.02^{a}	3.47 ± 0.01^{ab}	0.021	0.047	ns
Volatile acidity $_{\text{acetic acid}}$ (g/L)	0.57 ± 0.04^{ab}	0.69 ± 0.04^{b}	0.51 ± 0.04^{a}	0.54 ± 0.00^{a}	0.016	0.045	ns
Titratable acidity $_{\text{tartaric acid}}$ (g/L)	5.79 ± 0.03	5.38 ± 0.19	5.87 ± 0.29	5.53 ± 0.29	ns	ns	ns
Final nitrogen $_{\text{YAN}}$ (mg/L)	31.50 ± 4.95	29.75 ± 2.47	33.25 ± 2.47	36.75 ± 2.47	ns	ns	ns
Total SO_2 (mg/L)	29.44 ± 1.81	24.32 ± 1.81	26.88 ± 1.81	25.60 ± 0.00	ns	0.045	ns
Ethanol (% vol)	11.38 ± 0.18	11.13 ± 0.18	11.13 ± 0.18	11.00 ± 0.35	ns	ns	ns
Final reducing sugar (g/L)	24.31 ± 5.88	24.66 ± 0.98	25.70 ± 3.43	21.71 ± 0.49	ns	ns	ns

Values with different letters (a,b) in the same row are significantly different according to Tukey test ($P<0.05$); ns - indicates no significant difference

The pH of mead varied from 3.46 to 3.53, being lower than the pH of honey-must. The pH and volatile acidity values were the parameters most influenced by the strain and the condition (free or immobilised cells); each strain displayed higher pH values and higher volatile acidity in fermentations with immobilised cells compared to those performed by free cells. In both conditions the pH values were lower for strain ICV D47. Volatile acidity in mead fermented by the strain QA23 in free and immobilised form was 0.57 and 0.69 g/L acetic acid, respectively. These values were significantly higher than those obtained in mead

produced by the strain ICV D47, irrespective of the condition (0.51 – 0.54 g/L of acetic acid). Slightly lower values of volatile acidity have already been reported in mead obtained with free cells (Pereira et al., 2013). The use of high fermentations volumes in this work probably modified the fermentation conditions, which may affect yeast growth or induced physiological stress, and therefore modulate the accumulation of acetic acid (Ugliano and Henschke, 2009). Total SO_2 was lower in mead fermented with immobilised cells. In sum, the yeasts strains behaved similarly when submitted to similar conditions and thus no significant interaction between strain and condition was verified, for the parameters tested.

The concentrations of sugars, glucose and fructose, and fermentation products, ethanol, glycerol and acetic acid, at the end of fermentations are shown in Table 6.2.

Table 6.2. Concentration of sugars, glycerol, acetic acid and ethanol of mead fermented by *S. cerevisiae* QA23 and ICV D47 with free cells (F) or immobilised cells (I) and significance of the factors strain (S) and condition (C) according to two-way ANOVA.

	QA23 F	QA23 I	ICV D47 F	ICV D47 I	Significance		
					Strain	Condition	S x C
Glucose (g/L)	1.78 ± 0.53	1.72 ± 0.03	1.84 ± 0.23	1.69 ± 0.07	ns	ns	ns
Fructose (g/L)	2.72 ± 0.06^{a}	2.66 ± 0.16^{a}	3.67 ± 0.14^{b}	3.05 ± 0.14^{a}	0.002	0.021	0.040
Glycerol (g/L)	5.23 ± 0.19^{b}	5.14 ± 0.08^{ab}	5.07 ± 0.21^{ab}	4.43 ± 0.25^{a}	0.032	ns	ns
Acetic acid (g/L)	0.30 ± 0.02^{b}	0.39 ± 0.03^{c}	0.21 ± 0.01^{a}	0.29 ± 0.01^{b}	0.001	0.002	ns
Ethanol (%)	9.63 ± 0.05	10.12 ± 0.06	10.36 ± 0.15	9.54 ± 0.78	ns	ns	ns

Values with different letters (a-c) in the same row are significantly different according to Tukey test (P<0.05); ns - indicates no significant difference

The concentrations of fructose consumed and glycerol and acetic acid produced were mostly dependent on the strain. The strain ICV D47 has consumed less fructose than the strain QA23, resulting in mead with higher residual fructose (3.67 and 3.05 g/L for free and immobilised cells, respectively). On the other hand, the strain QA23 produced higher amounts of glycerol and acetic acid, either in free or immobilised form. Consumption of fructose and production of acetic acid were dependent on the yeast cells condition: yeast free cells consumed less fructose and conversely, immobilised cells produced more acetic acid. The values of acetic acid ranged from 0.21 to 0.39 g/L, values lower than volatile acidity. This result confirms that volatile acidity comprises a group of volatile organic acids, including acetic acid which comprises about 90% of volatile acids, and others acids like propionic and hexanoic acids (Swiegers et al., 2005). Accordingly, the interaction between the strain and cell condition was statistical significant, in respect to fructose concentration.

Mead aromatic compounds

Alcoholic fermentation by yeast result not only in ethanol and carbon dioxide production but also in a complex mixture of flavour-active by-products. The concentrations of volatile compounds in mead produced by strain QA23 and ICV D47 in immobilised and free form are shown in Table 6.3, together with the ANOVA results for the factors "strain (S)" and "condition (C)". A total of twenty-seven compounds were identified and quantified, including alcohols, esters, volatile phenols and volatile fatty acids.

The alcohols were quantitatively the largest group of volatile compounds and 3-methyl-1-butanol was the major compound in all mead studied. Alcohols are, from a quantitative point of view, the major group of volatile compounds produced by yeast during alcoholic fermentation (Ugliano and Henschke, 2009). Concentrations of alcohols below 300 mg/L add a desirable level of complexity to wine, whereas concentrations that exceed 400 mg/L can have a detrimental effect (Swiegers et al., 2005). The strain had a significant effect on the production of methanol and 3-ethoxi-1-propanol, instead the condition influenced the production of five alcohol compounds (methanol, 2-methyl-1-propanol, 2-methyl-1-butanol, 3-methyl-1-butanol, and 3-(methylthio)-1-propanol). Even so, four alcohol compounds present a significant interaction between the two factors, strain and condition (2-methyl-1-propanol, 2-methyl-1-butanol, 3-methyl-1-butanol and 3-(methylthio)-1-propanol). The alcohol 3-ethoxi-1-propanol was produced significantly in lower amounts in fermentation conducted with strain ICV D47 irrespective the condition. Similar results have already been obtained for this strain in mead under other fermentation conditions (Pereira et al., 2013 and 2014b). In general, independently of the strain, the immobilization of yeast cells led to lower concentrations of 2-methyl-1-propanol, 2-methyl-1-butanol, 3-methyl-1-butanol.

Table 6.3. Concentration of volatile compounds of mead fermented by *S. cerevisiae* QA23 and ICV D47 with free cells (F) or immobilised cells (I) and significance of the factors strain (S) and condition (C) according to two-way ANOVA.

	QA23 F	QA23 I	ICV D47 F	ICV D47 I	Significance		
					Strain	Condition	S x C
Alcohols (mg/L)							
methanol	3.25 ± 0.66^{ab}	4.82 ± 1.01^{b}	1.09 ± 0.24^{a}	4.01 ± 0.24^{b}	0.028	0.007	ns
1-propanol	32.28 ± 6.04	41.87 ± 1.14	40.15 ± 8.98	65.37 ± 18.23	ns	ns	ns
2-methyl-1-propanol	15.73 ± 2.04^{b}	14.74 ± 0.69^{ab}	18.27 ± 1.05^{b}	10.77 ± 0.13^{a}	ns	0.007	0.018
2-methyl-1-butanol	13.36 ± 0.47^{bc}	11.04 ± 1.79^{ab}	16.61 ± 0.32^{c}	8.79 ± 0.35^{a}	ns	0.002	0.015
3-methyl-1-butanol	104.72 ± 3.49^{ab}	99.65 ± 14.05^{ab}	125.18 ± 9.75^{b}	78.79 ± 6.16^{a}	ns	0.017	0.034
2-phenylethanol	24.59 ± 5.19	24.78 ± 6.48	35.50 ± 3.29	21.64 ± 5.68	ns	ns	ns
3-ethoxy-1-propanol	0.18 ± 0.01^{b}	0.16 ± 0.01^{b}	0.02 ± 0.01^{a}	0.009 ± 0.004^{a}	0.000	ns	ns
3-(methylthio)-1-propanol	0.0136 ± 0.0003^{a}	0.010 ± 0.004^{a}	0.04 ± 0.01^{b}	0.005 ± 0.001^{a}	ns	0.008	0.016
Esters (mg/L)							
ethyl acetate	34.28 ± 0.81	50.07 ± 11.83	28.02 ± 1.61	38.03 ± 2.94	ns	0.041	ns
ethyl butyrate	0.15 ± 0.04	0.167 ± 0.003	0.08 ± 0.01	0.16 ± 0.06	ns	ns	ns
isoamyl acetate	1.49 ± 0.16	1.13 ± 0.33	1.16 ± 0.40	1.12 ± 0.27	ns	ns	ns
ethyl hexanoate	0.47 ± 0.05	0.31 ± 0.10	0.37 ± 0.13	0.44 ± 0.08	ns	ns	ns
ethyl lactate	0.07 ± 0.01	0.05 ± 0.01	0.08 ± 0.03	0.04 ± 0.01	ns	ns	ns
ethyl octanoate	0.92 ± 0.21	0.45 ± 0.01	0.80 ± 0.08	0.71 ± 0.23	ns	ns	ns
ethyl decanoate	nd	0.24 ± 0.02a	0.82 ± 0.25b	0.19 ± 0.01a	0.013	ns	0.009
ethyl phenylacetate	0.017 ± 0.005^{b}	0.007 ± 0.001^{ab}	0.013 ± 0.001^{ab}	0.006 ± 0.001^{a}	ns	0.010	ns
2-phenylethyl acetate	0.74 ± 0.22	0.51 ± 0.09	0.67 ± 0.13	0.37 ± 0.06	ns	ns	ns
ethyl dodecanoate	0.08 ± 0.03	0.007 ± 0.002	0.12 ± 0.07	0.007 ± 0.004	ns	0.030	ns
Volatile phenols (µg/L)							
4-vinylguaiacol	128.11 ± 27.76	53.15 ± 3.72	122.07 ± 38.86	59.03 ± 4.80	ns	0.015	ns
4-vinylphenol	183.67 ± 28.65	157.34 ± 8.41	179.66 ± 5.62	139.85 ± 16.90	ns	ns	ns
Volatile fatty acids (µg/L)							
isobutyric acid	25.80 ± 0.71	19.41 ± 0.43	40.12 ± 19.15	17.06 ± 4.90	ns	ns	ns
butanoic acid	20.89 ± 2.68	15.07 ± 3.03	29.13 ± 11.76	15.52 ± 5.44	ns	ns	ns
hexanoic acid	714.12 ± 95.56	713.94 ± 14.99	757.47 ± 98.22	769.58 ± 296.92	ns	ns	ns
octanoic acid	3224.03 ± 282.58	2825.68 ± 293.58	3094.58 ± 758.90	2817.21 ± 335.32	ns	ns	ns
decanoic acid	1263.80 ± 71.73	1178.30 ± 178.95	1081.01 ± 354.72	1126.96 ± 204.77	ns	ns	ns
dodecanoic acid	3.48 ± 1.80^{ab}	10.69 ± 1.26^{c}	2.55 ± 0.90^{a}	8.72 ± 1.69^{bc}	ns	0.003	ns
Carbonyl compounds (mg/L)							
acetaldehyde	15.80 ± 2.25^{b}	3.63 ± 0.28^{a}	12.72 ± 2.33^{b}	4.32 ± 0.53^{a}	ns	0.001	ns

Values with different letters (a-c) in the same row are significantly different according to Tukey test (P<0.05); ns - indicates no significant difference; nd - indicates not detected

The esters were the second group of quantified volatile compounds. The production of esters by the yeasts during fermentation can have a significant effect on the fruity flavours in wine (Bartowsky and Pretorius, 2009; Swiegers et al., 2005). Comparatively with alcohols, less number of esters showed significant differences among strains or conditions. The strain QA23 in free form has not produced ethyl decanoate ester, whereas the strain ICV D47 produced it in higher concentration in free than in immobilised form, leading to a significant interaction S x C. A significant effect of the condition was observed in the production of ethyl acetate, ethyl phenylacetate and ethyl dodecanoate. Ethyl acetate was the major ester found in mead. Roldán et al. (2011) observed that ethyl acetate concentration is related to acetic acid content, so higher volatile acidity led to higher ethyl acetate concentration. The concentration of ethyl acetate varied from 28.02 to 50.07 mg/L, being higher in mead produced with immobilised cells. Similar results have already been reported in mead (Pereira et al., 2014b) or in white wine (Genisheva et al., 2012) produced with immobilised cells. The reverse was observed for ethyl phenylacetate and ethyl dodecanoate, i.e., lower concentrations were found in mead obtained by immobilised cells.

Volatile phenols are formed by decarboxylation of hydroxycinnamic acid precursors, *p*-coumaric, caffeic and ferulic acids (Boulton et al 1996). These acids have also been detected in chestnut, sunflower, lavender and acacia honeys (Tomás-Barberán et al., 2001). Vinylphenols, particularly 4-vinylguaiacol and 4-vinylphenol, are responsible for producing a pharmaceutical odour (Swiegers et al., 2005). Higher concentrations of these two phenols were detected in fermentations with free cells. Similar results have been recently reported in wine assays using immobilized cells (Genisheva et al 2014b). The volatile phenol present in higher concentrations in mead was 4-vinylphenol, however only the production of 4-vinylguaicol was significantly influenced by the cell condition.

Medium Chain Fatty Acids (MCFA) are produced through the lipid metabolism by yeast and are usually associated with unpleasant aromas, such as fatty, sweat, rancid or cheese (Ferreira et al., 2000). Six MCFA were identified and quantified in all fermentations. Octanoic acid was the main MCFA found in all mead, as already reported in a previous work in the same type of beverage (Pereira et al., 2013). The concentration of dodecanoic acid showed significant differences among yeast cell conditions, being higher in fermentations performed by immobilised cells.

Acetaldehyde is the major carbonyl compound found in wine, contributing to flavour with aroma descriptors such as ‘bruised apple’ and ‘nutty’ but can also be associated with oxidation off-flavors at high concentrations (Swiegers et al., 2005; Ugliano and Henschke, 2009). The concentration of acetaldehyde was dependent on yeast cell conditions; higher amounts were detected in mead produced with free cells, approximately 13 and 16 mg/L for strain ICV D47 and QA23, respectively. These results are in agreement with the ones reported in wine by Genisheva et al. (2014b) and Tsakiris et al. (2004), who also observed higher amounts of acetaldehyde in wines produced with free cells. Nevertheless, the values found in this work are above those previously reported in mead (Pereira et al., 2013 and 2014b; Roldán et al., 2011).

Odour activity values

The odour activity values (OAVs) were determined in order to evaluate the contribution of each volatile compound to the mead aroma. Only the compounds with an OAV > 1 contribute individually to the beverage aroma (Guth, 1997). However, in wine, compounds with an OAV less than 1, may also contribute to the aroma because the additive effect of similar compounds (Vilanova et al., 2010). The compounds with more influence (OAV > 1) on mead aroma are presented on Table 6.4. From the twenty seven volatile compounds quantified, only fourteen were above their perception threshold, and therefore were potential contributors to mead’s aroma.

Among the esters, a total of 7 compounds presented OAV > 1, being isoamyl acetate, ethyl hexanoate and ethyl octanoate the most aromatic and thus may contribute to the beverage with fruity/floral characteristics (Guth, 1997; Moreno et al., 2005), although, no significant differences in their concentrations among strain or cell condition was observed (Table 6.3).

The alcohols, 3-methyl-1-butanol and also 2-phenylethanol were present above its odour threshold, particularly in mead produced with free cells. 2-phenylethanol is generally a positive contributor to wine aroma, being characterised by a pleasant rose-like aromatic alcohol (Swiegers et al. 2005).

Three MCFA, usually associated with unpleasant aromas of fatty, rancid and cheese, were detected in all mead above their odour perception threshold, being octanoic acid in higher concentrations in mead produced with free cells. Although, according to the ANOVA,

no statistical significant differences were observed between conditions (Table 6.3). They are precursors of esters associated with fruity character, like ethyl octanoate (a fruity, sweet aroma,) which exhibited the highest OAV and the strain QA23 in free form produced the highest value (OAV = 183.50).

Table 6.4. Odour activity values (OAV) of volatile compounds of more influence on the aroma of mead fermented by *S. cerevisiae* QA23 and ICV D47 with free cells (F) or immobilised cells (I).

Compounds	Odour descriptor [a]	Odour threshold (µg/L)	QA23 F	QA23 I	ICV D47 F	ICV D47 I
3-methyl-1-butanol	Cheese; nail polish	30 000	3.49	3.32	4.17	2.63
2-phenylethanol	Roses; flowery	14 000	1.76	1.44	2.54	1.55
ethyl acetate	Solvent; nail polish	12 300	2.79	4.07	2.28	3.09
ethyl butyrate	Fruity; sweet	20	7.40	8.35	4.20	8.13
isoamyl acetate	Banana	30	49.70	37.54	38.63	37.31
ethyl hexanoate	Fruity; aniseed	14	33.60	22.42	26.68	31.34
ethyl octanoate	Fruity; sweet	5	**183.50**	**89.93**	**159.69**	**141.31**
ethyl decanoate	Pleasant; soap	200	---	1.18	4.1	---
2-phenylethyl acetate	Flowery; roses	250	2.95	2.03	2.67	1.49
4-vinylphenol	Almond shell	180	1.02	---	1.00	---
hexanoic acid	Cheese; sweaty	420	1.70	1.70	1.80	1.83
octanoic acid	Fatty; rancid	500	6.45	5.65	6.19	5.63
decanoic acid	Fatty; soap	1 000	1.26	1.18	1.08	1.13
acetaldehyde	Fresh; green	500	31.60	7.26	25.45	8.65

[a] Odour descriptors reported in the literature (Culleré et al., 2004; Czerni et al., 2008; Escudero et al., 2004; Meilgaard, 1975; Siebert et al., 2005).

The volatile phenol, 4-vinylphenol, was detected in concentrations above its perception threshold (OAV > 1) in mead produced with free cells. Volatile phenols play a minor role in the aroma of most wines, unless their concentration is above certain limits seems to depreciate the aroma of wine by masking the fruity character, and giving phenolic off-flavors (Baumes, 2009).

Acetaldehyde was one of the most aromatic compounds, and their contribution was particularly relevant in mead produced with free cells, 3 to 4 times higher compared to immobilized cells (Table 6.4). The OAVs showed that mead produced by free cells presented a more interesting aroma profile. The opposite was observed in a previous work about mead

production with the same strains free and immobilised but in fermentations in smaller volumes (Pereira et al., 2014b).

To obtain a more simplified view of the relationship of mead with their volatile composition, a Principal Component Analysis (PCA) was performed using the fourteen aroma compounds with OAV >1 (Figure 6.1).

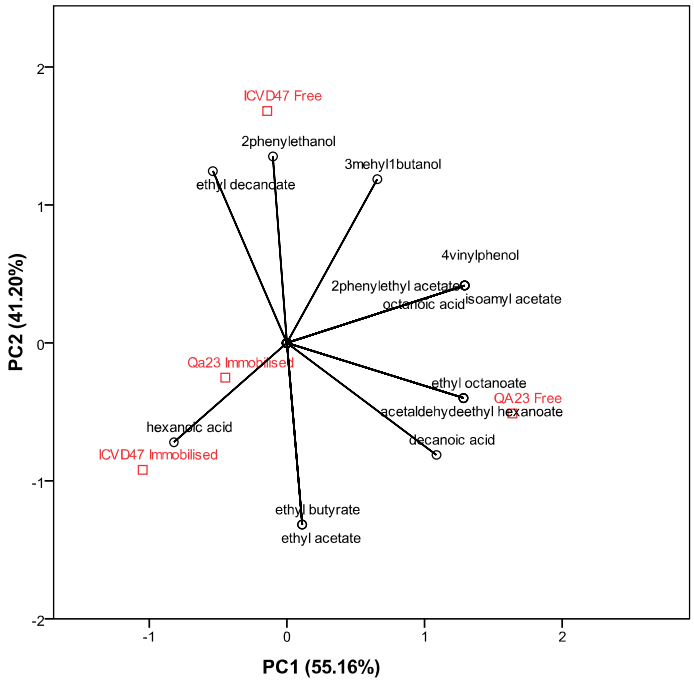

Figure 6.1. Principal component analysis (PCA) plot, using the values of volatile compounds concentrations quantified in mead obtained by the two strains, QA23 and ICV D47, with free or immobilised cells.

The approach allowed identifying the volatiles compounds that better discriminate the different mead. The first two principal components, PC1 and PC2, accounted for 96.36% of total variance, 55.16% and 41.20%, respectively. The first component, PC1, was characterized by higher levels of isoamyl acetate, 2-phenylethylacetate, 4-vinylphenol, octanoic acid, ethyl hexanoate, ethyl octanoate and acetaldehyde. For the second principal component, PC2, the volatile compounds 2-phenyltehanol and ethyl decanoate showed the highest and positive values, while ethyl acetate and ethyl butyrate contributed to the negative side of the same principal component. PC1 discriminated mead produced with free or immobilised cells of the strain QA23 and PC2 discriminated mead produced by strain ICV D47. In general mead produced with free cells were characterized by compounds associated with pleasant aromas: ethyl octanoate (sweet fruity), acetaldehyde (green leaves, fresh) and ethyl hexanoate (apple, aniseed, fruity) for strain QA23 and 2-phenylethanol (roses, flowery),

ethyl decanoate (pleasant, soap, fruity) and 3-methyl-1-butanol (cheese, nail polish) for strain ICV D47. Instead, mead produced with immobilised cells, located at negative values of PC1 and PC2, were mainly correlated with off-flavour compounds: ethyl acetate (solvent, nail polish), ethyl butyrate (fruity, sweet) and hexanoic acid (sweaty, cheese).

Mead sensory analysis

Mead samples were subjected to a sensory characterization in order to evaluate the effect of strain and their form (free or immobilised) in their aroma and flavour. The analysis was performed by a panel of 16 semi-trained tasters using a total of 10 sensory attributes: two for appearance (color and turbidity), three for taste (sweet, sour and astringency) and five for aroma (fruity, honey, vegetable, alcohol and chemical).

For interpreting the results, PCA was applied to identify the aroma descriptors that better discriminated mead obtained by the two strains mentioned above (Figure 6.2).

The first two principal components accounted for 92.13% of total variance. PC1, which accounted for 79.62% of total variance, clearly discriminated mead produced with free or immobilised cells. The first component, PC1, was high positively correlated with turbidity and astringency and so, the appearance and taste were the greatest contributors to discriminating mead produced by free yeast cells. This mead was also correlated to the following sensory aroma attributes: acid, vegetal and honey. Mead produced with immobilized cells located at the negative side of the PC1, were correlated to the attributes of color and alcohol. PC2 accounted for 12.51% of variance and the attributes of alcohol and vegetal showed high and positive values and sweet and fruity contributed to the negative side of same PC. In fact, the results obtained in sensory analysis reflect the degree of clarification of mead produced by immobilized cells, correlated with appearance (color), whereas mead obtained with free cells were correlated with the attribute turbidity. The strain and the condition had a significant effect on volatile acidity and therefore on acetic acid concentration, which were higher in mead produced by the strain QA23 in immobilised form (Tables 6.1 and 6.2). However, the sensory analysis showed that the attribute acid was more perceptible in mead obtained with the strain QA23 in free form. In general, the overall appreciation revealed that tasters showed preference for mead produced with free cells.

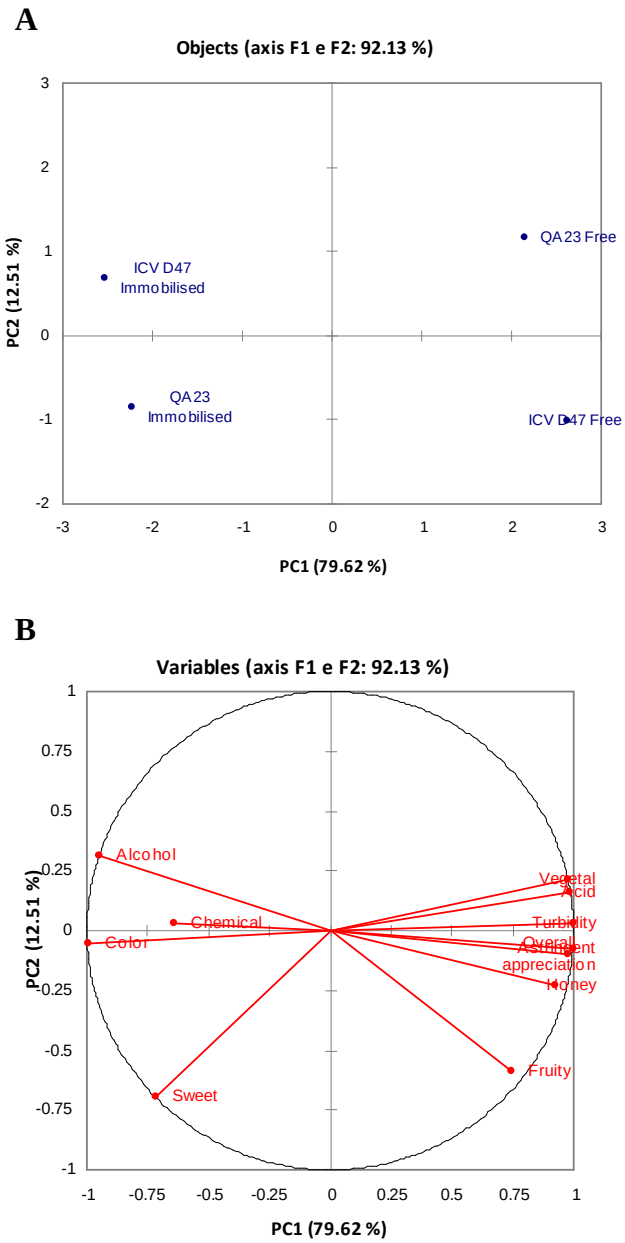

Figure 6.2. Principal component analysis (PCA) plot of mead obtained by the two strains, QA23 and ICV D47, with free or immobilised cells (A) and scores of sensory descriptors (B).

The results of the PCAs for the 14 volatile compounds with OAV>1 and the results obtained from the sensory analysis indicate that the aroma descriptors that discriminate for mead produced by yeast free cells are: acid, vegetal and fruity (Figure 6.2). These descriptors are associated with volatile compounds that better characterized mead, namely, ethyl octanoate, acetaldehyde, ethyl hexanoate, 2-phenylethanol and ethyl decanoate (Figure 6.1). Moreover, mead produced by free cells of the strain QA23 was more aromatic (compounds with higher OAV) than that produced by the strain ICV D47 (Table 6.4 and Figure 6.1). However, that difference was not perceptible by the taster panel. In contrast with mead

produced with free cells of the strain ICV D47, the one obtained with immobilised cells was characterized by volatile compounds associated with unpleasant aroma, such as, hexanoic acid and ethyl acetate (Figure 6.1), which was noticeable in sensory analysis with the aroma attributes of alcohol and chemical (Figure 6.2). Regarding the strain QA23 there is not a so apparent distinction between mead produced with free or immobilised cells, as for strain ICV D47. For instance, the attribute sweet was correlated with mead produced with immobilised cells (Figure 6.2), which were characterized by the unpleasant aroma compounds, such as ethyl acetate, ethyl butyrate and hexanoic acid (Figure 6.1).

Conclusions

This study is one of the first approaches combining volatile composition and sensory properties of mead. Two strains, QA23 and ICV D47, in free or immobilised form were used to produce four different meads. The strain and yeast cell conditions had significant effect on some characteristics of the final product, such as final pH, volatile acidity, fructose degradation, and volatile compounds formation. Only fourteen volatile compounds out of the twenty seven quantified, were above their perception threshold, and therefore were potential contributors to mead aroma. This work reveals a correlation between the volatile characteristics and sensory properties of mead. The sensory analysis allowed to distinguish mead produced with free and immobilised cells; high scores were given to mead obtained with free cells compared to those obtained by immobilised yeast. The strain ICV D47 behaved differently in terms of aroma compounds formation in free or immobilised cells; differences were less pronounced in the strain QA23. In general, yeast cell conditions (free or immobilised) had more influence than the strain on the sensory characteristics of final product. Despite some off-flavour compounds detected in mead produced with free cells, they were overall more appreciated by the taste panel.

Considering the results obtained in respect to sensory properties of mead and to have a better understanding on the correlation between volatile composition and sensory properties, further studies focused on sensory quality should be performed.

CHAPTER 7

Final Considerations and Perspectives

Mead is a traditional alcoholic beverage made of honey which is mainly produced at a homemade level. Given the demand for this kind of beverage in the last decade much researchers has been focused on solving some of the problems associated to its production. So, this work intended to optimize mead quality by improving yeast growth and yeast fermentative performance through the supplementation of honey-must, the application of higher inoculum size or by using immobilized yeast cells. It was also intended to establish a potential correlation between the sensory properties and the volatile aroma composition of mead produced with free or immobilized cells.

The research developed during this work, allowed to achieve the following main conclusions:

- the supplementation of honey-must with salts and/or vitamins did not improve yeast growth or yeast fermentative performance;
- dark honey composition was able to provide all the essential minerals and vitamins for fermentation;
- increasing the inoculum size resulted in significant time savings in the fermentation process;
- minor differences were detected in the fermentation length and fermentation rate among fermentations conducted with free or immobilized cells;
- higher concentrations of viable cells were achieved in immobilized systems;
- the entrapment agent had no negative effects on mead production, since no remarkable differences were observed among fermentations conducted with free or immobilized cells;
- the phenomenon of cell leakage was not reduced by increasing alginate concentrations nor by the use of double-layer alginate-chitosan immobilization;
- the honey-must supplementation with salts increased the SO_2 concentration of mead produced by the strain QA23;
- volatile acidity of meads increased in fermentations with higher inoculums size and in those produced with immobilized cells;
- residual nitrogen, from 30 to 40 mg/L, remained in all mead at the end of fermentations, probably corresponding to the amino acid proline which is not assimilable by yeasts;

- some residual sugar remained in mead, corresponding to non-fermentable sugars, such as trehalose, isomaltose, saccharose and melezitose;
- the formation of volatile compounds in concentrations above their perception threshold was particularly pronounced in fermentations with low pitching rates and with immobilized cells;
- the alcohols were the major group of volatile compounds quantified in all mead;
- the esters isoamyl acetate, ethyl octanoate and ethyl hexanoate, and acetaldehyde were the major powerful odorants found in mead, contributing to its fruity character;
- the concentrations of these compounds was enhanced in mead produced with low inoculum sizes and in mead produced with immobilized cells;
- the concentrations of undesirable volatile compounds were higher in fermentations with immobilized cells compared to fermentations conducted with free cells;
- the sensory analysis allowed to distinguish mead produced with free and immobilized cells;
- yeast cell conditions (free or immobilized) had more influence than the strain on the sensory characteristics of final product;
- despite of some off-flavour compounds detected in mead produced with free cells, high scores were given to this mead compared to that obtained by immobilized yeast.

The overall results achieved in this work showed that the availability of vitamins and salts in the honey-musts was not a limiting factor for fermentation. The application of lower inoculums size seems to be more suitable for mead production since it improves the formation of desirable aroma compounds and sensory analysis revealed that mead produced with free cells was overall more appreciated.

Considering the results obtained in this research work, future studies should be focused on continuing to improve the fermentation process and consequently, the quality of mead. So, in future, some work may pass through:

- to perform a sensory analysis on mead produced with honey-must supplemented with salts and/or vitamins in order to understand the effect of its addition on sensory quality;
- to evaluate the use of fed-batch and continuous fermentation processes in mead production. These processes may emerge as an alternative, since, although the batch system has been the most used, it shows some deficiencies, in particular those related to low concentrations of reagents and the slow process of final product purification.

Also, and was far as we know, the fed-batch cultures have not been studied for mead production;

- to study the use of mixed cultures for the production, once previous studies demonstrate that the use of multiple strains have a positive effect on mead fermentation;
- to improve the sensory quality of mead with the addition of honey or fruit juices at different stages of fermentation, because the studies available on sensory analysis of mead revealed that the tasters usually prefer a beverage more sweet. Due to the high osmotic pressure involved in these fermentations, the effect of the addition should be assessed not only on the quality of mead but also on yeast growth and yeast fermentative performance.

Finally, the production of mead derivatives seems to be an opportunity to explore. Although there is some research in this field, studies are very scarce. Considering the growing interest of consumers in gourmet products, including vinegars, mead could be used as raw material for the elaboration of quality ones.

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