JOSHUA JOHANNES VAN MULLEM

SCREENING OF VOLATILE ORGANIC COMPOUNDS PRODUCED BY YEASTS FROM A BRAZILIAN YEAST CULTURE COLLECTION (CCMA) AND SELECTION OF YEASTS FOR THE PRODUCTION OF MEAD

LAVRAS-MG
2019

JOSHUA JOHANNES VAN MULLEM

SCREENING OF VOLATILE ORGANIC COMPOUNDS PRODUCED BY YEASTS FROM A BRAZILIAN YEAST CULTURE COLLECTION (CCMA) AND SELECTION OF YEASTS FOR THE PRODUCTION OF MEAD

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós Graduação em Microbiologia Agrícola, área de concentração em Microbiologia Agrícola, para a obtenção do título de Doutor.

Orientadora

Profa. Dra. Rosane Freitas Schwan

Coorientadores

Prof. Dr. Disney Ribeiro Dias

Dr. Leonardo de Figueiredo Vilela

LAVRAS – MG 2019

Ficha catalográfica elaborada pelo Sistema de Geração de Ficha Catalográfica da Biblioteca Universitária da UFLA, com dados informados pelo(a) próprio(a) autor(a).

Van Mullem, Joshua Johannes.
Screening of volatile organic compounds produced by yeasts from a Brazilian yeast culture collection (CCMA) and selection of yeasts for the production of mead / Joshua Johannes Van Mullem. - 2019.
91 p. : il.

Orientador(a): Rosane Freitas Schwan.
Coorientador(a): Disney Ribeiro Dias, Leonardo de Figueiredo Vilela.
Tese (doutorado) - Universidade Federal de Lavras, 2019.
Bibliografia.

1. Non-conventional yeasts. 2. Yeast culture collection. 3. Mead. I. Schwan, Rosane Freitas. II. Dias, Disney Ribeiro. III. Vilela, Leonardo de Figueiredo.

JOSHUA JOHANNES VAN MULLEM

SCREENING OF VOLATILE ORGANIC COMPOUNDS PRODUCED BY YEASTS FROM A BRAZILIAN YEAST CULTURE COLLECTION (CCMA) AND SELECTION OF YEASTS FOR THE PRODUCTION OF MEAD

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós Graduação em Microbiologia Agrícola, área de concentração em Microbiologia Agrícola, para a obtenção do título de Doutor.

APROVADA em 27 de fevereiro de 2019.

Prof. Dr. Disney Ribeiro Dias	UFLA
Prof. Dr. Nelson Manuel Viana da Silva Lima	UMINHO
Dra. Maria Gabriela da Cruz Pedrozo Miguel	UFLA
Profa. Dra. Cíntia Lacerda Ramos	UFVJM

Profa. Dra. Rosane Freitas Schwan
Orientadora

LAVRAS – MG

2019

ACKNOWLEDGEMENTS

It is my pleasure to extend my deep gratitude to the following people and organizations, whose support of various kinds have helped enable this research.

To the Universidade Federal de Lavras (UFLA) for the opportunity to pursue my doctorate.

To the professors of the post-graduate program in Agricultural Microbiology for all they have taught me and for their commitment to the quality of the program.

To Prof. dr. Rosane Freitas Schwan for having welcomed me at UFLA, for the opportunity to work on this project and for her advices and trust.

To Prof. Dr. Disney Ribeiro Dias, dr. Leonardo de Figueiredo Vilela, Prof. Dr. Nelson Lima, Prof. Dr. Ismael Mancilha, dr. Maria Gabriela da Cruz Pedrozo Miguel and Prof dr. Cíntia Lacerda Ramos for the fruitful discussions.

To Rose, Cidinha and Ivani for their invaluable assistance.

To all my colleagues, in particular Angelica and Claudia.

To Jing, Pedro, Ludmila, Julio and Stephen.

Muito obrigado!

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001.

ABSTRACT

Yeast culture collections harbor great genetic and phenotypical diversity that can be explored to identify strains that demonstrate an exceptional suitability for specific biotechnological applications. One such application is bio-flavoring, in which yeasts are utilized to enhance the aroma profile of fermented foods and beverages. In the first part of this study, a screening of the production of volatile aroma compounds by yeast isolates (n = 63) from the CCMA (Coleção de Culturas da Microbiologia Agrícola) was carried out. The purpose was to select strains that have potential for application as bio-flavoring agents in mead fermentations. Strains were grown on culture medium and volatile organic compounds were identified by gas chromatography – mass spectrometry. This screening identified several strains that produced aroma compounds of interest that represent floral and fruity aromas. Clustering of yeasts by aroma profile enabled the selection of strains that produce aroma compounds in amounts that are relevant for food fermentations (n = 19). While several strains that produced attractive aroma profiles were not able to grow on honey must, seven yeast strains were able to produce a sweet, low-alcoholic mead (45-168 g/l of residual sugar, 3.8-6.5% ethanol v/v) with distinct aroma profiles. In the second part of this study, four strains (*Saccharomyces cerevisiae CCMA 1523*, *Pichia jadinii CCMA 0160*, *Torulaspora delbrueckii* CCMA 1524 and *Kluyveromyces lactis* CCMA 1518) were selected for larger scale mead fermentations, as single cultures or co-cultures of *S. cerevisiae* and each of the other yeasts individually. Among other parameters, consumption of sugars, production of ethanol and organic acids in the meads were determined. An untrained tasting panel evaluated the intensity of selected taste and flavor attributes and hedonic appreciation. In both single strain and mixed strain fermentations with *S. cerevisiae*, meads produced with either *T. delbrueckii* or *K. lactis* had a roughly three-fold higher content of honey-aroma compound phenethyl acetate. Meads produced with *T. delbrueckii* had the highest acceptance rate, followed by meads produced with *K. lactis*. Also meads produced with mixed cultures containing these strains had higher acceptance rates than those produced with only *S. cerevisiae*. Moreover, the sensory panel expressed a preference for low-alcoholic meads with high residual sweetness. The results demonstrate the potential of non-conventional yeasts to improve the aroma profile and sensory qualities of low-alcoholic meads.

Key-words: Non-conventional yeasts, yeast culture collection, Bio-flavoring, Alcoholic fermentations, Yeast starter cultures, Mead

TABLE OF CONTENTS

1 INTRODUCTION 8
1.1 General introduction 8
1.2 Scope of this research 10
2 THEORETICAL BACKGROUND 12
2.1 Yeast biodiversity: interconnected ecological and metabolic strategies 12
2.2 Aroma production pathways 14
2.3 Biotechnological applications of non-conventional yeasts 18
2.4 Yeast starter cultures in the production of alcoholic beverages 19
2.5 Production of mead 21
2.6 Composition of honey, nutrient supplementation and their influence on mead 24
3 FINAL CONSIDERATIONS 27
4 REFERENCES 28
5 METHOD DEVELOPMENT FOR GAS CHROMATOGRAPHY - MASS SPECTROMETRY 37
5.1 Comparison of solvent extraction versus solid phase micro-extraction and automatization of data analysis 37
5.2 Development of a protocol for data analysis 40
5.3 Materials and methods 42
5.4 References 43
Article 1: SCREENING OF VOLATILE ORGANIC COMPOUNDS PRODUCED BY YEASTS FROM A BRAZILIAN CULTURE COLLECTION (CCMA) AND SELECTION OF STRAINS WITH POTENTIAL AS BIO-FLAVORING AGENTS 44
Abstract 44
1 Introduction 45
2 Materials and methods 46
3 Results and discussion 48
4 Conclusions 56
5 References 57
Appendix 1 60
Appendix 2 62
Appendix 3 63
Appendix 4 64

ARTICLE 2: PRODUCTION OF LOW-ALCOHOLIC MEAD WITH NON-CONVENTIONAL YEASTS IMPROVES AROMA PROFILE AND SENSORY QUALITY 65
Abstract......65
1 Introduction......66
2 Materials and methods 67
3 Results and discussion 70
4 Conclusions......81
5 References......81
Appendix 1......86
Appendix 2......87
Appendix 3......89

1 INTRODUCTION

1.1 General introduction

Public service microbial cultural collections provide indispensable services to the scientific community and society at large. By preserving and providing access to microbial strains and associated information they allow researchers to revisit past studies and incorporate greater biodiversity in future experiments. Microbial culture collections facilitate the quest to find exceptional strains that advance the capability and profitability of biotechnological processes.

As pointed out elsewhere, research results in the area of microbiology cannot be verified independently if strains are not publicly available, and therefore these results might lack validity (Janssens et al., 2010). Not only does the failure to deposit strains to a culture collection for long-term preservation weaken the foundations of microbiology as a science, it also wastes the biotechnological potential that these strains may have.

This rings particularly true for the application of food fermentation research. While scientists have studied fermented foods of a diverse nature and geographic origin over the course of decades, the majority of the micro-organisms that were isolated in these studies are not known to be deposited in culture collections and are presumed lost (Daniel and Prasad, 2010).

In recognition of these issues, the Laboratory of Microbial Physiology and Genetics of the Federal University of Lavras (UFLA), which specializes in the microbiology of fermented foods and silage, has a long-standing policy of preserving strains that are part of its published research. Over the course of 20 years more than 2000 strains of bacteria and yeasts have been added to its collection. These have been made available to the public through the Culture Collection of Agricultural Microbiology (CCMA), which is registered under number 1083 at the World Federation of Culture Collections since 2014 (World Federation for Culture Collections, n.d.).

Most strains in the CCMA collection have been isolated in Brazil. While Brazil is a biodiversity hotspot for animal and plant species, the whole of Latin America was labelled a ‘virgin territory’ in terms of yeast biodiversity in the not-too-distant past (Boekhout, 2005), as most major culture collections are based in North-America and Western Europe. More recently, the Brazilian Microbiome Project (BMP) has coordinated various projects aiming to

close this gap by mapping the microbial biodiversity of various ecological niches in the country (Pyrlo et al., 2014).

In this light, the CCMA collection is a valuable archive of biodiversity for this underexplored region of the world. Furthermore, most strains have been isolated from agricultural substrates and may therefore be equipped with the right stress tolerances and nutrient utilization profiles for innovations in the area of green biotechnology.

A promising potential application of yeasts in the CCMA collection lies in the area of bio-flavoring of fermented foods. Bio-flavoring is a key research interest in non-conventional yeast studies, since strains of several non-*Saccharomyces* (often referred to as non-conventional) yeast species have been demonstrated to produce desirable aroma-active compounds in significantly higher concentrations than *Saccharomyces cerevisiae* reference strains (Ravasio et al., 2018; Steensels and Verstrepen, 2014). These yeasts have also received interest from the brewing industry, as they may contribute additional complexity to the sensory profile of fermented beverages.

In this research project, the suitability of yeast strains from the CCMA collection (most of which are non-conventional yeasts) for application in bio-flavoring of alcoholic beverages is investigated by focussing on the production of mead.

Mead is an alcoholic beverage obtained by fermentation of a solution of bee honey, water and potentially other additives such as salts and/or hops. It is produced in a relatively simple process by diluting honey up to 3-4x with water and fermenting it with a yeast starter culture or natural microflora. After the inoculation, fermentation usually takes place at temperatures between 22 and 27 °C (Ramalhosa et al., 2011). Fermentation can take days, weeks or months to complete depending on the composition of the must, the chosen yeast and conditions. The final ethanol concentration is up to 18% by volume (Pereira et al., 2015).

The reason that mead was chosen as the focus of this research project is in part the economic importance of honey for the Brazilian economy. Worldwide, Brazil is among the top-10 exporting countries of honey, with exports totalling over 27.000 tons in 2017. This honey was sold at an average price of $4.48 / kg (ABEMEL, 2018). Since a kilogram of honey could be used to produce at least three liters of mead, which could likely be sold at a higher price, mead production seems an attractive opportunity for Brazilian beekeepers to add value to their produce.

While there currently is little domestic mead production in Brazil, Brazilian consumers are used to drinking sweet, honey-flavored spirits (conhac and cachaça) and may therefore find a honey-based alcoholic drink appealing.

Another reason why mead is chosen as the focus of this project, is that application of non-conventional yeasts in mead is an understudied area. While several studies have been performed to select strains of *Saccharomyces cerevisiae* or *Saccharomyces bayanus* and optimize fermentation parameters (Jung et al., 1999; Mendes-Ferreira et al., 2010; Pereira et al., 2009), little is known about the application of non-conventional yeasts in the production of mead.

Mead seems a suitable product for the application of non-conventional yeasts since many non-conventional yeast strains have been shown to produce high amounts of floral and honey aroma compounds (Gamero et al., 2016; Gutiérrez et al., 2018), which may be a good complement to the aroma profile of meads.

1.2 Scope of this research

In the preparatory phase of this research, an attempt was made to establish a high-throughput protocol to facilitate the aroma screening by gas-chromatography coupled with mass-spectrometry. Successfully establishing a high-throughput research protocol would enable the screening of a larger number of strains from the culture collection. A comparison was made between two methods of sample preparation: a solvent-extraction protocol (combined with an autosampler) and a solid phase microextraction (following a manual procedure). In addition, an algorithm was developed to assist in GC-MS data analysis that speeds up data processing and reduces analyst bias. The results of this comparison and a description of the algorithm are provided in Chapter 5.

The main part of this research project is dedicated to the selection of yeasts from the CCMA collection with attractive aroma profiles that are suitable for the production of alcoholic beverages.

First, an aroma screening is performed based on culture medium fermentations. This provides an overview of the diversity of aroma profiles among strains in the collection and gives insight in their distribution among genera and species.

Second, strains with attractive aroma profiles are applied in small-scale mead fermentations. This step enables the selection of yeast strains that grow well on honey must and produce meads with distinct aroma profiles. The results of the aroma screening and selection process are reported in Article 1, along with a discussion of the basis for the observed differences.

Finally, it is evaluated if the selected yeast strains are indeed suitable starter cultures that produce attractive meads. To this end, the four most promising strains are applied in larger

scale fermentations. Non-*Saccharomyces* yeasts are applied both as single strain starter cultures and in co-culture with *Saccharomyces cerevisiae*. Fermentation performance and aroma production are evaluated for each of the produced meads. Furthermore, a sensory panel is asked to evaluate intensity of selected taste and flavour attributes in the beverages as well as hedonic appreciation.

The results of this experiment, which are reported in Article 2, reveal which strains are most suitable for mead production. They also provide general insights into the performance of non-conventional yeast starter cultures in mead production.

2 THEORETICAL BACKGROUND

2.1 Yeast biodiversity: interconnected ecological and metabolic strategies

Yeasts (unicellular fungi) are a highly diverse group of micro-organisms. Yeast taxonomists have recognized nearly 1500 species, grouped among 149 different genera (Kurtzman et al., 2010). This number is certain to increase over time as many of the earth's biomes remain unexplored and it is estimated that millions of fungal species are still undiscovered (Blackwell, 2011). Furthermore, yeast taxonomy is constantly up for revision as new insights into phylogenetic relationships among yeasts are sometimes at odds with previous phenotype-based classifications (Kurtzman et al., 2008).

Far from being the "original" ancestors of fungi, yeasts have branched off from multicellular fungi on separate occasions (Hittinger et al., 2015). For this reason, they can be found among various fungal taxa and display distinct biological characteristics, such as reproduction mode. Yeast lineages are separated by up to 550 million years of divergent evolution, counting from the moment Ascomycota and Basidiomycota split (Langkjær et al., 2003).

Different habitat pressures have led to an accumulation of adaptations over time that gave rise to genetically diverse and highly specialized yeasts. Yeasts inhabit every major biome on earth: from oceans and soil to grape skin and the human gut (Péter and Rosa, 2006). However, as heterotrophs yeasts always depend on the availability of organic carbon sources.

One result of these selective pressures is that yeast phenotypes demonstrate varying abilities to resist physiological and chemical stress factors. For example, yeasts can be capable of growing at extremely low temperatures (cryophilic) such as *Saccharomyces kudriavzevii* (Tronchoni et al., 2014) or at high temperatures (thermophilic) such as *Kluyveromyces marxianus* (Banat et al., 1992). Other examples of specialized yeasts include acidophiles (capable of growing at low pH) and osmophiles (low water activity).

However, yeast adaptations to specific habitats go far beyond those general factors. Many natural environments are inhospitable because an essential nutrient is depleted, or water is scarce, or noxious compounds make growth of most micro-organisms impossible. Under such circumstances the environment selects micro-organisms for efficiency of utilization of the limiting nutrients and resistance against specific chemical stressors (Cray et al., 2013; Lievens et al., 2015).

In practice such selective pressures may lead to differentiation in carbohydrate and amino acid transporters, benefiting transporters with affinities that are well adjusted to the availability of nutrients in the environment. For example, strains of *Saccharomyces cerevisiae* utilized by the beer industry possess a maltose transporter allele with improved affinity for maltotriose that enable to it to ferment this sugar in beer wort (Gallone et al., 2018).

Similarly, yeasts – even from the same species – might produce a different range of extracellular hydrolytic enzymes (Belda et al., 2016; Buzzini and Martini, 2002). Such hydrolytic enzymes can be instrumental in freeing up nutrients stored in macromolecules that the cell cannot take up directly. It has also been claimed that pectinase production by yeasts plays a role in plant pathogenicity, and protease production in animal pathogenicity as they can be used to penetrate host tissues (Collmer and Keen, 1986; Naglik et al., 2003).

An additional source of variation among yeasts is their carbohydrate metabolism. Even if the major metabolic pathways are preserved among yeasts, they differ in the range of sugars that they are able to assimilate and/or ferment, as well as transport mechanisms of sugars into the cell (Flores et al., 2000). Yeast carbohydrate metabolism is also subject to various regulation mechanisms, such as the Pasteur effect (the observation that glucose is consumed more rapidly under anaerobic conditions), the Kluyver effect (yeasts can assimilate, but not ferment some sugars) and the Crabtree effect (the occurrence of alcoholic fermentation under aerobic conditions).

The Crabtree effect is demonstrated by *Saccharomyces cerevisiae* as well as some related species in the *Saccharomycetaceae* family (Dashko et al., 2015). The metabolic strategy followed by these yeasts has been dubbed the “make-accumulate-consume” strategy. Since the glycolytic pathway supports a higher flux than the citric acid cycle, ATP can be generated at a higher rate by performing alcoholic fermentation, thus boosting its growth rate. While Crabtree-positive yeasts typically have a high tolerance for ethanol, the accumulation of ethanol can kill competing micro-organisms in the habitat. When all sugars have been converted into ethanol, the yeast can switch to utilizing ethanol as a carbon and energy-source to harvest its remaining energy (Piškur et al., 2006).

Whereas the rationale for production of primary metabolites such as ethanol is fully understood, this does not always apply to secondary metabolites, including many volatile organic compounds (VOC’s) that are produced by yeasts. Large screenings of culture collections have shown that yeasts produce similar VOC’s across species, such as esters, acids and alcohols, but in substantially different amounts (Gamero et al., 2016).

Some volatile compounds may simply be unintended by-products of metabolism. This is likely the case for compounds such as higher alcohols that are products of the Ehrlich pathway, by which yeasts break down branched-chain and aromatic amino acids (Hazelwood et al., 2008).

In some cases, volatile compounds may be involved in quorum sensing, a process by which yeasts track their own cell density by producing and detecting signal molecules, in order to induce metabolic and morphological transformations above certain thresholds (Sprague and Winans, 2006).

Esters form another class of metabolites of which the biological role remains unknown. Some hypotheses that aim to explain ester production by yeasts focus on biochemical reasons (such as a potential role in controlling the rigidity of the cell membrane) (Saerens et al., 2010); others suggest an ecological motive. There is evidence that yeasts produce aroma-active esters to attract pollinators that aid in their distribution to sugar-rich environments (Asahina et al., 2008) or to limit the growth of competing micro-organisms (Masoud et al., 2005). It is not unthinkable that esters primarily fulfil a biochemical function, but that some high producing yeasts have hacked its production to gain ecological benefits, much like *S. cerevisiae* has exploited its capacity to produce ethanol to gain a competitive edge.

2.2 Aroma production pathways

During alcoholic fermentations, yeasts produce a variety of compounds that can contribute to the aroma profile of the final product (Styger et al., 2011; Pires et al., 2014). Primary metabolites are compounds that are derived from metabolic pathways that are considered essential for yeast growth and cell division and include ethanol, acetic acid, carbon dioxide and other products of central carbon metabolism. Metabolites that are derived from pathways that are not considered essential for growth are referred to as secondary metabolites, and include esters and higher alcohols (Hirst et al., 2016).

In most fermentation processes, sugars serve as the main carbon- and energy source for yeasts. This means that yeasts depend on sugar catabolism to obtain the ATP that drives anabolic reactions and cellular growth. A schematic overview of pathways involved in sugar catabolism and their end-products is shown in Figure 1.

After glucose is converted into pyruvate through the glycolytic pathway there is a branching point: under aerobic conditions, it can enter the TCA cycle to generate more

energy after respiration. Under anaerobic conditions, it can be converted to ethanol to restore the redox balance; the cell needs to turn the NAD^+ that was converted to NADH during glycolysis back into NAD^+, so that glycolysis can continue (Van Dijken and Scheffers, 1986). Crabtree-positive yeasts, such as *S. cerevisiae*, are known for their ability to produce ethanol under aerobic conditions as well (Piskur et al., 2006).

Two other major compounds that are products of sugar catabolism are acetic acid and glycerol. Acetic acid is a precursor of acetyl CoA, which is a building block used in lipid biosynthesis (Liu et al., 2017). It is produced from pyruvate by oxidation of the intermediate acetaldehyde, while reduction of acetaldehyde yields ethanol (Dzialo et al., 2017). Glycerol is produced in a side-chain of the glycolytic pathway and is involved in regulation of osmotic pressure inside the cell (Scanes et al., 1998).

A detailed review on carbon metabolism by yeasts (of both *Saccharomyces* and *non-Saccharomyces* species) is given by Compagno et al. (2014).

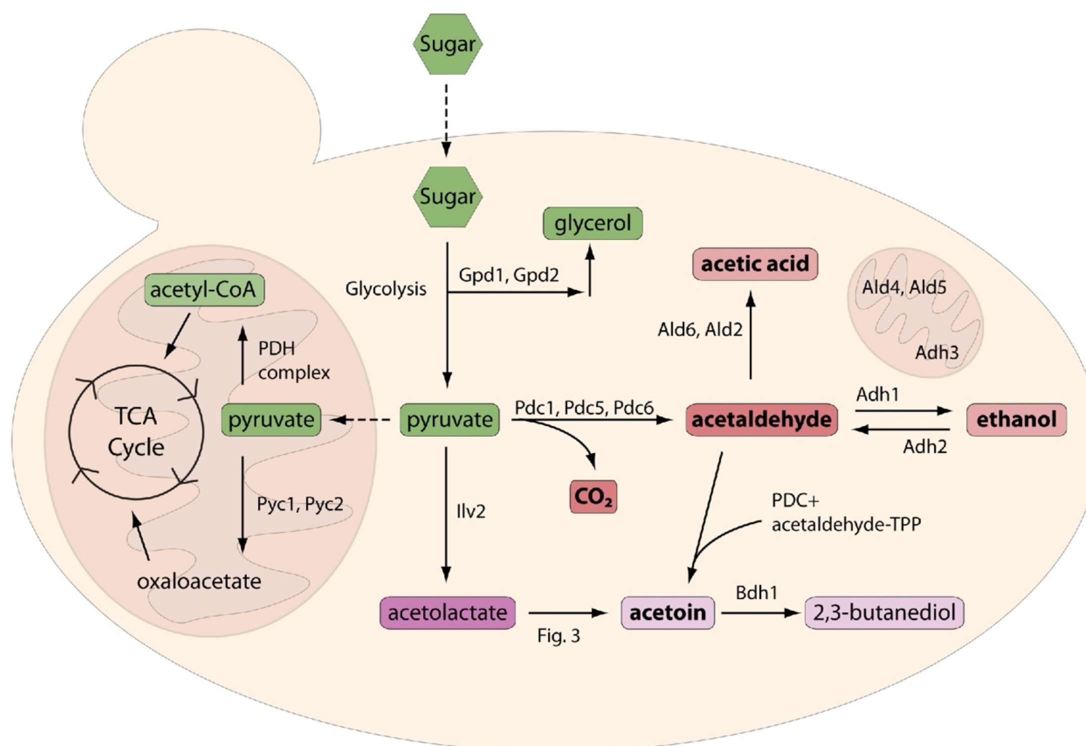

Figure 1. Production of primary metabolites by yeasts, indicating the most important enzymes and localization inside the cell. Source: Dzialo et al. (2017), licensed under CC-BY**.**

Yeast cells require nitrogen for the biosynthesis of amino acids, the building blocks of proteins. This can be obtained from a variety of sources, including other amino acids and ammonium. The cells convert most of their available nitrogen sources into glutamate, which is an important intermediate for the biosynthesis of new amino acids. Glutamate is produced

by removing the ammonium group from another amino acid and attaching it to α-keto glutarate (Broach, 2012).

For some amino acids, including branched chain amino acids, aromatic amino acids and methionine, the removal of the nitrogen group results in waste products that are excreted from the cell. After the ammonium group is removed from these amino acids, the remaining carbon skeleton (an α-keto acid) is broken down in a few steps. The pathway by which this happens is known as the Ehrlich pathway, named after its discoverer (Ehrlich, 1907). The steps of this pathway are shown in Figure 2.

The higher alcohols and acids that are end-products of this pathway are also known as 'fusel alcohols' and 'fusel acids' due to their past association with bad liquor. However, more recently it was recognized that some higher alcohols can give positive contributions to the aroma profile of fermented foods. A detailed review of this pathway is provided by Hazelwood et al. (2008).

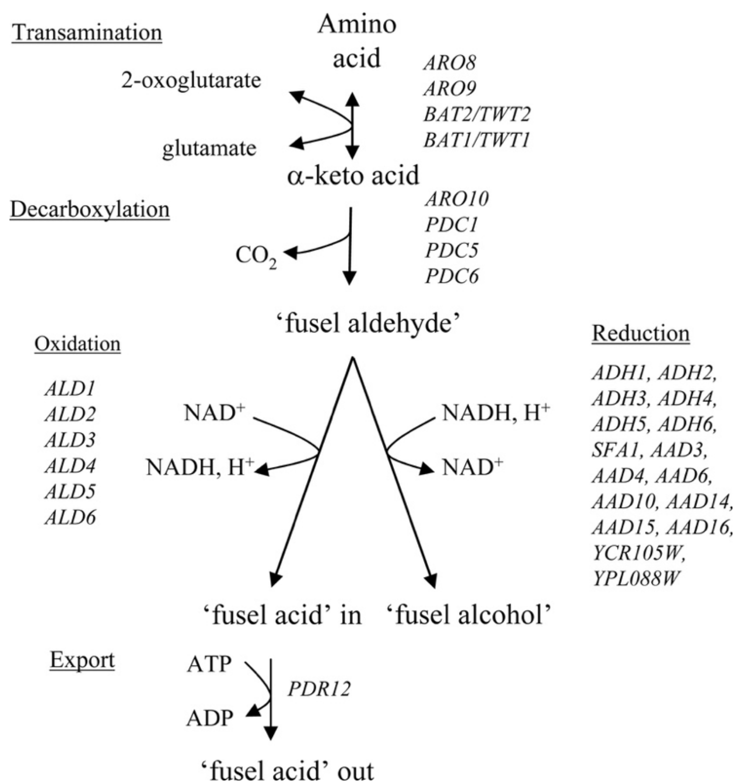

Figure 2. Production of higher alcohols through the Ehrlich pathway, indicating the most important enzymes and intermediate steps. Source: Hazelwood et al. (2008), reprinted with permission.

Higher alcohols can serve as precursors for esters. From a quantitative point of view, esters are minor metabolites. However, they are important to the aroma of fermented food products owing to their low flavour thresholds. Two important categories of esters are acetate esters and medium-chain fatty acid (MCFA) ethyl ester (Saerens et al., 2010). These groups of esters are made by different enzymes and might be subject to different regulation mechanisms. A schematic overview of their biosynthesis is presented in Figure 3.

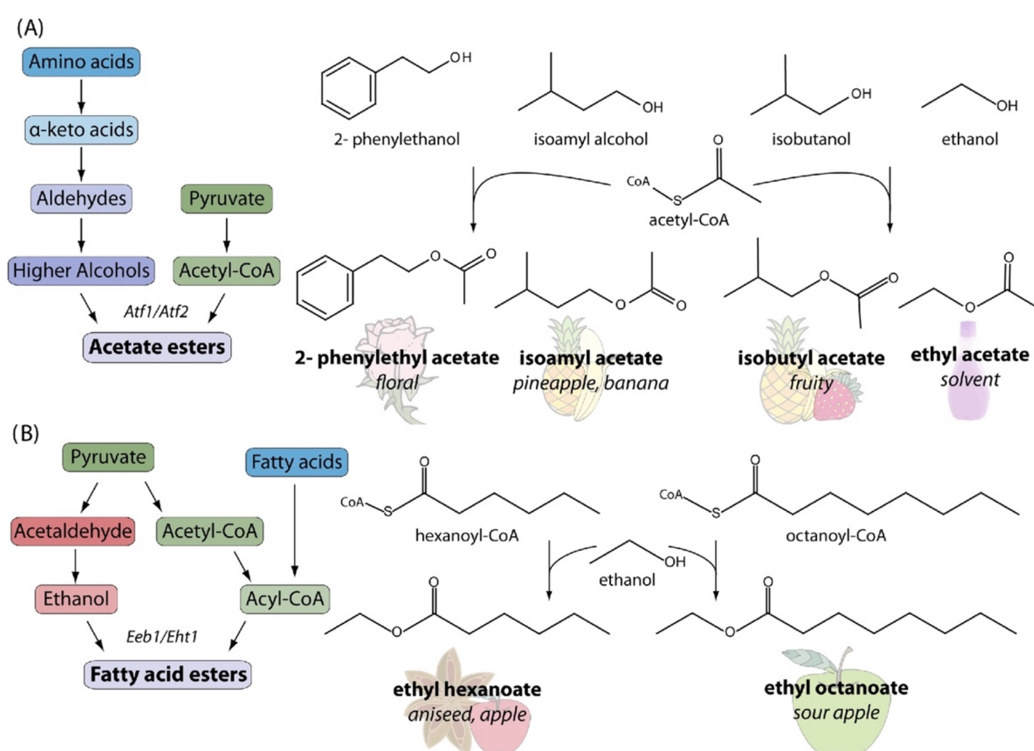

Figure 3. Production of acetate esters (A) and fatty acid esters (B) by yeasts, indicating the most important enzymes, precursors and associated aromas. Source: Dzialo et al. (2017), licensed under CC-BY.

Acetate esters are formed from acetyl CoA and an alcohol, which can be ethanol or one of the higher alcohols (acetyl CoA is the oxidation product of pyruvate and part of the citric acid cycle). The acetyl-transferases Atf1p and Atf2p play a major role in their formation in *S. cerevisiae*, although strains in which both encoding genes were deleted still produced some acetate esters; this shows that not all ester synthases have been identified yet (Verstrepen et al., 2003).

The production of acetate esters seems to be governed more by the expression of acetyl-transferase encoding genes than by the concentration of their acetyl-CoA or alcohol

substrates. It has been demonstrated that over-expression of the AFT1 gene can increase the production of some esters up to 180 times. Moreover, the specific acetate ester profile can differ between strains with different alleles of these genes (Verstrepen et al., 2003).

The MCFA esters are made from ethanol and acyl-CoA, in which the acyl group can have different chain lengths (acyl-CoA's are intermediates in the breakdown of lipids). The formation of these esters is catalyzed by a different group of enzymes, the acyl-CoA: ethanol O-acyl transferases. Contrary to what is observed for acetate esters, the formation of MCFA esters seems to be influenced more by the concentration of acyl-CoA precursors than by the expression of the responsible genes (Saerens et al., 2006).

2.3 Biotechnological applications of non-conventional yeasts

Yeasts are the main workhorses of modern biotechnology, exceeding other groups of micro-organisms in terms of production volume as well as generated value. Economically, the most important applications of yeast in biotechnology include the production of ethanol from biomass, fermented foods and enzymes for the food, chemical and pharmaceutical industry. Other valuable uses of yeasts can be found in the area of environmental protection, including biological control of pests and degradation of pollutants (Johnson and Echavarri-Erasun, 2011).

While *S. cerevisiae* is by far the most widely employed yeast species in most of the applications, the use of so called non-conventional yeasts can offer significant advantages. In part, such opportunities arise from the known weaknesses and limitations of *S. cerevisiae*. For example, *Hansenula polymorpha* is capable of producing ethanol from xylose in lignocellulosic biomass, while *S. cerevisiae* is not (in its native form) (Suwannarangsee et al., 2010). This property increases the efficiency of biofuel production.

Another benefit of many other non-conventional yeasts is that they are Crabtree-negative and do not produce ethanol under aerobic conditions; this is an undesirable by-product of *S. cerevisiae* in some biotechnological processes such as enzyme or biomass production (Wagner and Alper, 2016).

Other potential applications result from comparative strengths of non-conventional yeast species. For example, they may possess phenotypes that produce high concentrations of desirable metabolites or have high stress tolerance. The yeast *Yarrowia lipolytica*

demonstrates a unique ability to produce polyols, lactones, surfactants and emulsifiers and as such finds applications in the food industry (Zinjarde, 2014).

Beyond native capabilities of yeasts, they may lend themselves for biotechnological applications by virtue of being suitable host systems for genetic engineering. The species *H. polymorpha*, *Kluyveromyces lactis, Pichia pastoris* and *Y. lipolytica* are particularly promising in this context because they possess promoters that allow for tight regulation of biotechnological processes in which they are employed (Wagner and Alper, 2016).

Furthermore, as yeasts are rarely pathogenic to humans, utilization of most yeast species does not cause any safety concerns, as is more frequently the case with bacteria.

2.4 Yeast starter cultures in the production of alcoholic beverages

Yeasts are responsible for producing alcoholic beverages, by converting fermentable sugars into ethanol and carbon dioxide. Louis Pasteur discovered the biological origin of alcoholic fermentation in 1857. Pasteur's discovery made it possible to radically change the way yeasts are applied in food fermentations, such as beer, wine and mead production. Soon after it, the industry shifted from fermentations based on natural flora or back-slopping (using part of a previous ferment), to defined, pure starter cultures.

Traditionally, wine fermentations were carried out by the natural microflora of the grapes. This included a variety of yeast species from different genera (Romano et al., 2003). In the beer industry, back-slopping was the most common way to start a fermentation. Successful back-slopping cultures were exchanged among breweries, helping to spread effective strains (Stewart and Russell, 1986).

In natural fermentation processes, microbial communities go through a dynamic process in which various species thrive for a period of time. In wine making, the process often ends up being dominated by strains of *S. cerevisiae* once the ethanol concentration reaches 3-4 volume percent (Pretorius, 2000).

A number of factors explain why this species is so competitive: it produces ethanol, is ethanol tolerant, and grows fast, even at the low levels of oxygen that are present during winemaking (Piškur et al., 2006).

Ever since Pasteur's discovery, the industry has sought to increase its control over yeast fermentations by using defined starter cultures. Hansen, of the Danish Carlsberg breweries, was among the first to isolate pure yeast cultures from beer. He re-pitched four of his isolates in fresh wort and found that only one of them was able to produce a satisfactory beer. This

yeast, known as *Saccharomyces carlsbergensis (*or *S. pastorianus)*, was taken into production in 1883. The species is still the world's major lager yeast (Stewart and Russell, 1986).

Nowadays defined, single strain starters are applied in practically all industrial beverage fermentations. This has made the product's quality more constant, it has improved the efficiency of the process and reduced the risk of spoilage (Hui, 2004). Indeed, applications of mixed starter cultures has led to inconsistent results and the result of mixing two yeasts with distinct 20lavour profiles may have unpredictable outcomes (Holt et al., 2018).

To answer why *Saccharomyces* species became the most important yeast for the industry, we can identify different factors:

- Potential starters are isolated from finished fermented products and then screened for their ability to reproduce it;
- *Saccharomyces* species often dominate in those finished products;
- The industry prefers to use pure strains, instead of co-cultures, because this makes their results more constant.

Non-Saccharomyces yeasts (also called 'non-conventional yeasts') were initially seen as undesirable because they were held responsible for spoilage of wine: they were associated with high levels of volatile acidity and off-flavors. When it was discovered that also 'good' wines could result from fermentations in which non-Saccharomyces species attained high cell numbers, their role in aroma formation was investigated more closely and it was acknowledged that they could make positive contributions and add complexity to the product (Jolly et al., 2013).

Over the last 15 years, non-conventional yeasts have received a great deal of attention for their ability to produce diverse 20lavour profiles (Buzzini et al., 2003; Moreira et al., 2005). Plenty of such studies have concluded that certain non-Saccharomyces yeast strains are high producers of some VOC's, mainly higher alcohols and esters, that are associated with attractive floral and or fruity aromas (for example isoamyl acetate, which has a banana odor, or phenethyl alcohol which has a rose aroma) (Gamero et al., 2016; Ravasio et al., 2018). Therefore, non-conventional yeasts may contribute pleasant flavors to fermented products and increase sensory complexity.

Some research into non-conventional yeasts focused on the application of non-conventional yeasts as starter cultures in the prodctuon of specific alcoholic beverages. In a screening of 38 yeast strains from several genera, Viana et al. (2008) found non-Saccharomyces strains that were particularly apt at producing acetate esters; *Hanseniaspora*

osmophila was considered a good candidate for wine mixed starter cultures. Duarte et al. (2012) found that co-culturing *S. cerevisiae* with *Pichia caribbica* produced cachaça (a Brazilian spirit) with improved characteristics. More studies on this topic are reviewed by Steensels and Verstrepen (2014).

Nonetheless, only a few non-conventional yeasts have found their way to the market. The South-African wine yeast blends Viniflora® Harmony.nsac and Viniflora® Melody.nsac include *Torulaspora delbrueckii* (Van Breda et al., 2013). Refermentation of beer with yeasts from the genus *Brettanomyces spp.* is popular among adventurous draft brewers and produces beer with a characteristic style (Fromson, 2012). Of course, non-conventional yeasts still play a role in many of the world's spontaneously fermented products.

Encouraged by these results, multiple companies and research groups now investigate more innovative applications of non-Saccharomyces yeasts. One idea that is discussed in literature is to ferment beer and wine with respiratory yeasts, to produce low-alcoholic beverages (Gonzalez et al., 2013). Non-conventional species may also lower the ethanol content of alcoholic beverages because they are less efficient at converting sugars into ethanol (Ciani et al., 2016).

Ultimately, finding novel yeast isolates with improved performance is a promising way for the alcoholic beverage industries to diversify their products. It can be seen as an alternative for genetically improving strains through genetic engineering, hybridization or laboratory evolution. Robot-assisted hybridization of *S. cerevisiae* strains was used by Snoek et al. (2015) to improve ethanol tolerance and by Mertens et al. (2015) to improve aroma production. So far, legal issues and consumer perceptions limit the application of GMO-yeasts (Fleet, 2008).

If society's attitude towards GMO's changes in the future, this won't make knowledge of non-conventional yeasts obsolete: mining their diversity of enzymes and regulation strategies can then become a source of inspiration for brewing strain engineers.

2.5 Production of mead

Mead is a fermented beverage produced with honey. It is considered one of the oldest fermented drinks in human history with archaeological evidence pointing back to China in the 7th millennium bC as its first known origin (McGovern et al., 2004). Mead was among the most consumed alcoholic beverages in the Roman Empire and remained popular in Northern

Europe and Russia well into the Middle Ages (Vidrih and Hribar, 2007). Recently it is making a modest comeback with several hundreds of small-scale producers bringing a great variety of mead styles to the market (Kettmann, 2016).

The Brazilian legislation defines mead as a drink that contains between 4 and 14% of alcohol by volume at 20 *ºC* obtained by fermentation of a solution of bee honey, salts and water (Decrete 6871 from the 4th July, 2009). In US legislation, meads are considered 'agricultural wines' and may not contain alcohol in excess of 14% by volume, while only water and hops are permitted as additions to the honey (26 U.S.C. 5387).

The honey is diluted with water to reduce osmolarity and allow growth of yeasts. The proportions of honey and water used vary greatly among styles and recipes, from 120 g/l to about 600 g/l. Formulations that use a large amount of honey take a longer time to complete (up to several months) and may have larger amounts of residual sweetness.

Naturally, fermentation performance also depends on the chosen yeast. Hobbyist mead brewers frequently use commercial wine strains of *S. cerevisiae* from suppliers such as Lallemand, Wyeast and Red Star. These strains are selected based on criteria that include its maximal ethanol tolerance, optimal growth temperature, aroma production and nitrogen requirements.

After the inoculation, fermentation usually takes place at temperatures between 22 and 27 °C (Ramalhosa et al., 2011) until most of the sugars are consumed and fermentation is no longer active. The final ethanol concentration is up to 18% by volume (Pereira et al., 2015), although depending on local legislation this amount may be too high for commercial use. Fermentation can take days, weeks or months to complete depending on the composition of the must, the chosen yeast and conditions. It is common for meads to have some residual sugars, and this is not always considered undesirable as it can help the meads retain more honey-like characteristics.

Upon completion, mead is typically clarified by chilling or chemical fining agents. This results in a transparent beverage that is more attractive to consumers. It can then be stored in bottles or aged on woods, if so desired for up to 10 years (Schramm, 2003).

A summary of experimental conditions that were used during mead production in previous scientific studies is provided in Table 1, focussing on must composition, inoculum, fermentation time and temperature. The rationale and effectivity of nutrient supplementation is discussed in the next sub-chapter. The information contained in this table reveals that the applied mead formulations and fermentation conditions were far from uniform, using a wide range of honey concentrations (roughly 20-40 °Brix), fermentation temperatures (15-30 °C)

and times (7-50 days). Various commercial *S. cerevisiae* wine strains were used as starter cultures.

Table 1. Experimental conditions used in previous studies on mead production

Honey variety	*Honey concen-tration*	*Supplements*	*Inoculum*	*Fermen-tation time*	*Fermen-tation temperature*	*Source*
Clover-wildflower	30g /100 ml	Diammonium phosphate, vitamin B1	*S. cerevisiae* C11-3 (internal collection, immobilized)	10 days	30 °C	Navrátil et al. (2001)
Cassava	35.7ml/ 100 ml	SO_2	*S. cerevisiae* (commercial baker's yeast)	21 days	25-26 °C	Ukpabi (2006)
Buckwheat	33.3ml/ 100 ml, 25ml/100 ml	Diammonium phosphate, citric acid	*S. cerevisiae* LOCK 105 (internal collection)	28 days	20-21 °C	Sroka and Tuzynski (2007)
Chestnut, lime, honeydew	25 °Brix	None	*Sacharomyces bayanus* R2 (commercial wine yeast, Lalvin)	24-39 days	15 °C	Vidrih and Hribar (2007)
Erica spp.	37g/100 ml	(i) none; (ii) potassium tartrate, malic acid, diammonium phosphate	*S. cerevisiae* UCD522 (UC Davis collection)	11-25 days	22 °C	Mendes-Ferreira et al. (2010)
Multifloral	21 °Brix	Potassium metabisulphite, tartaric acid, pollen	*S. cerevisiae* ENSIS-LE5 (commercial wine yeast, Tensum tecnologicas)	50 days	25 °C	Roldan et al. (2011)
Unspecified	39.5g/100 ml	Diammonium phosphate, vitamin B1, tartaric acid	*S. cerevisiae* ph.r. *bayanus* PB2002 (commercial wine yeast, AEB group)	15 days	20, 25 and 30 °C	Gomes et al. (2013)
Unspecified	24 °Brix	Diammonium phosphate, pollen	*S. cerevisiae* UVAFERM BC, FERBLANC AROM, and LALVIN QA23 (commercial wine yeasts)	20 days	30 °C	Hernandez et al. (2015)

Honey variety	*Honey concen-tration*	*Supplements*	*Inoculum*	*Fermen-tation time*	*Fermen-tation temperature*	*Source*
Castanea spp. and *Erica* spp.	37g/100 ml	Malic acid, diammonium phosphate	*S. cerevisiae* Lalvin QA23, ICV D47 (commercial wine yeasts, Lalvin)	7-10 days	25°C	Pereira et al. (2015)
Unspecified	33 °Brix	Acerola pulp, calcium carbonate, lactic acid	*S. cerevisiae* AWRI 796 (commercial wine yeast, Mauri Yeast)	12 days	30 °C	Amorim et al. (2018)
Produced by *Melipona scutellaris* bees	28 °Brix	Tartaric acid	*Saccharomyces cerevisiae* Fermol Reims Champagne (commercial wine yeast)	13 days	25 °C	Cavalcante da Silva et al. (2018)

2.6 Composition of honey, nutrient supplementation and their influence on mead

Key ingredients of mead are honey and water. The Codex Alimentarius (2001) defines honey as ‘the natural sweet substance produced by honey bees from the nectar of plants or from secretions of living parts of plants or excretions of plant sucking insects on the living parts of plants, 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.’

Most honey is indeed produced by honey bees of the genus *Apis* spp., however, various other genera of insects produce similar sweet substances (Crane, 1991).

For natural honeys produced by *Apis* spp., moisture contents between 7.99% and 21.60% have been reported. Its dry matter is mainly composed of fructose and glucose, which can be present in different ratios. For fructose, contents between 33.30 and 73.50 g/ 100 g have been reported; for glucose, this range is 21.00 to 38.30 g/ 100 g (da Silva et al., 2016). Other sugars are of minor importance.

The relevance of sugar composition for mead fermentations is that fructose is approximately twice as sweet as glucose (Moskowitz, 1970). Furthermore, yeasts of *S. cerevisiae* as well as most other yeasts prefer to consume glucose over fructose, which is fermented at a slower rate (Berthels et al., 2004). Fructose content may therefore influence

the time it takes to complete fermentation. Some fructophilic yeasts, such as *Zygosaccharomyces rouxii*, do metabolize fructose faster than glucose (Leandro et al., 2011).

Honey has a relatively low nitrogen content, which may negatively impact fermentation performance of yeasts. Honey has an average nitrogen content of 400 mg N / kg (Anklam, 1998). Upon dilution with water, this concentration drops and the amount of nitrogen may therefore not be enough to complete fermentation of a must rich in sugars. This situation is aggravated by the fact that more than 50% of the nitrogen in honey is in the form of proline, which is not readily utilized by *S. cerevisiae* (Mendes-Ferreira et al., 2010).

As grape must has a sugar composition that is similar to mead (about 240 g/l of total sugars, mainly glucose and fructose), some insights into wine fermentation may also apply to mead fermentation. A minimum nitrogen concentration of 140 mg N/ l is often cited as a requirement to complete fermentation of grape must (Bely et al., 1990) and addition of diammonium phosphate to exceed these levels is common practice in the wine industry, to avoid stuck fermentations.

Likewise, nitrogen supplementation with diammonium phosphate is recommended by home brewers of mead, in concentrations of approximately 0.4 g/l (corresponding to ~85 mg N/ l) (Schramm, 2003). Together with the ~100 mg N/l present in 4x diluted honey must this is likely enough to complete fermentation.

Addition of nitrogen in the form of diammonium phosphate at the beginning of mead fermentation has been shown to shorten the fermentation time (Pereira et al., 2015). Hobbyists often advocate staggered addition of nitrogen supplements (spread over the course of fermentation) but this has no basis in the scientific literature, which shows late-stage nitrogen additions during wine fermentations are largely ineffective (Beltran et al., 2005).

There is no consensus in the literature on whether nitrogen supplementation with diammonium phosphate increases the production of aroma compounds. Mendes-Ferreira (2010) found that nitrogen supplementation with diammonium phosphate in concentrations up to 300 mg N/l can have a positive influence on the formation of aroma compounds such as esters. However, Pereira et al. (2015) reported that esters were produced in higher amounts in absence of nitrogen supplementation (between both studies, comparable levels of nitrogen supplementations were used).

In mead, nitrogen supplementation is sometimes achieved with pollen, which are a source of the amino acids proline, aspartate, phenylalanine and glutamate (Roldan et al., 2011; Hernandez et al., 2015). Amino acid supplementation may influence aroma production, but this has not yet been studied in the context of mead. On synthetic media, supplementation

with amino acids has been shown to increase the production of related volatile compounds (Barbosa et al., 2012). The variety of amino acid profiles in natural products should also be considered as a factor that influences aroma composition, as has been shown on synthetic media mimicking grape varieties (Hernandez-Orte, 2012).

Besides diammonium phosphate, other frequently used additions include organic acids, vitamins and salts, but none of these were demonstrated to have a positive effect on mead fermentations (Mendes-Ferreira et al., 2010; Pereira et al., 2015). Apparently, honey must contains sufficient amounts of these nutrients for yeasts to complete fermentation.

A final aspect of honey that can influence the quality of mead, is the volatile compound composition. Monofloral honeys often possess more distinctive aroma profiles than multifloral honeys. Examples of important aroma compounds that are characteristic for specific honey varieties include 3-methyl-2-butanol in sunflower honey and cavravol in tilia honey and cis-linalool oxide for acacia honey (Juan-Borrás et al., 2014). Analysis of the volatile composition of honey has even been proposed as a manner to establish its authenticity (Radovic et al., 2001).

3 FINAL CONSIDERATIONS

As the yeast cultures that are present in the CCMA collection have been isolated from a range of agricultural substrates and include multiple different genera and species, they can be expected to display significant genotypical and phenotypical diversity.

Selecting the right yeast strains for specific applications, out of hundreds of available strains, requires large-scale screenings with rational experimental designs. Keeping in mind that each laboratory has its own opportunities and limitations with regards to laboratory infrastructure, the 'bottleneck' for efficient high-throughput screenings may be specific for each lab.

In the context of this study, priority was given to improving the extraction protocol, aiming to benefit from the presence of an automatic injector for liquid samples in gas-chromatography / mass spectrometry analyses.

Non-conventional yeasts have received significant academic interest as potential bio-flavoring agents since more than a decade. However, known examples of non-conventional yeast starter cultures being applied in artisanal and industrial production are so far limited. One reason might be that potent aroma compounds are produced by these yeasts in such amounts that overwhelm the sensory profile of a product such as wine or beer.

Instead of finding the right yeast for a product, one might also aim to find the right product for a yeast. As previous studies have found that many non-conventional yeasts produce high amounts of volatile compounds that are associated with rose aroma (such as phenethyl alcohol and its acetate ester) it may be considered if such floral aromas could blend in more harmoniously with a honey-based product such as mead than with a cereal-based product such as beer.

If the aroma compounds produced by these non-conventional yeasts do indeed intensify the existing floral aroma of honey in mead, this could enable the production of a sensorially acceptable mead that contains less honey and thus has fewer calories and a lower production cost.

4 REFERENCES

ABEMEL, 2018. Setor apícola em números. Retrieved September 21, 2018 from http://brazilletsbee.com.br/dados-setoriais.aspx

Amorim, T.S., Lopes, S. de B., Bispo, J.A.C., Bonafe, C.F.S., de Carvalho, G.B.M., Martínez, E.A., 2018. Influence of acerola pulp concentration on mead production by Saccharomyces cerevisiae AWRI 796. LWT 97, 561–569. https://doi.org/10.1016/J.LWT.2018.07.009

Anklam, E., 1998. A review of the analytical methods to determine the geographical and botanical origin of honey. Food Chem. 63, 549–562. https://doi.org/10.1016/S0308-8146(98)00057-0

Asahina, K., Pavlenkovich, V., Vosshall, L.B., 2008. The survival advantage of olfaction in a competitive environment. Curr. Biol. 18, 1153–5. https://doi.org/10.1016/j.cub.2008.06.075

Banat, I.M., Nigam, P., Marchant, R., 1992. Isolation of thermotolerant, fermentative yeasts growing at 52°C and producing ethanol at 45°C and 50°C. World J. Microbiol. Biotechnol. 8, 259–263. https://doi.org/10.1007/BF01201874

Barbosa, C., Mendes-Faia, A., Mendes-Ferreira, A., 2012. The nitrogen source impacts major volatile compounds released by Saccharomyces cerevisiae during alcoholic fermentation. Int. J. Food Microbiol. 160, 87–93. https://doi.org/10.1016/J.IJFOODMICRO.2012.10.003

Broach, J.R., 2012. Nutritional Control of Growth and Development in Yeast. Genetics 192, 73–105. https://doi.org/10.1534/genetics.111.135731

Belda, I., Ruiz, J., Alastruey-Izquierdo, A., Navascués, E., Marquina, D., Santos, A., 2016. Unraveling the Enzymatic Basis of Wine “Flavorome”: A Phylo-Functional Study of Wine Related Yeast Species. Front. Microbiol. 7, 12. https://doi.org/10.3389/fmicb.2016.00012

Beltran, G., Esteve-Zarzoso, B., Rozès, N., Mas, A., Guillamón, J.M., 2005. Influence of the Timing of Nitrogen Additions during Synthetic Grape Must Fermentations on Fermentation Kinetics and Nitrogen Consumption. J. Agric. Food Chem. 53, 996–1002. https://doi.org/10.1021/jf0487001

Berthels, N., Corderootero, R., Bauer, F., Thevelein, J., Pretorius, I., 2004. Discrepancy in glucose and fructose utilisation during fermentation by wine yeast strains. FEMS Yeast Res. 4, 683–689. https://doi.org/10.1016/j.femsyr.2004.02.005

Bely, M., Sablayrolles, J.-M., Barre, P., 1990. Automatic detection of assimilable nitrogen deficiencies during alcoholic fermentation in oenological conditions. J. Ferment. Bioeng. 70, 246–252. https://doi.org/10.1016/0922-338X(90)90057-4

Blackwell, M., 2011. The Fungi: 1, 2, 3 … 5.1 million species? Am. J. Bot. 98, 426–438. https://doi.org/10.3732/ajb.1000298

Boekhout, T., 2005. Gut feeling for yeasts. Nature 434, 449–451. https://doi.org/10.1038/434449a

Broach, J.R., 2012. Nutritional Control of Growth and Development in Yeast. Genetics 192, 73–105. https://doi.org/10.1534/genetics.111.135731

Buzzini, P., Martini, A., 2002. Extracellular enzymatic activity profiles in yeast and yeast-like strains isolated from tropical environments. J. Appl. Microbiol. 93, 1020–1025. https://doi.org/10.1046/j.1365-2672.2002.01783.x

Buzzini, P., Martini, A., Cappelli, F., Pagnoni, U.M., Davoli, P., 2003. A study on volatile organic compounds (VOCs) produced by tropical ascomycetous yeasts. Antonie Van Leeuwenhoek 84, 301–311. https://doi.org/10.1023/A:1026Ret064527932

Cavalcante da Silva, S.M.P., de Carvalho, C.A.L., Sodré, G. da S., Estevinho, L.M., 2018. Production and characterization of mead from the honey of *Melipona scutellaris* stingless bees. J. Inst. Brew. 124, 194–200. https://doi.org/10.1002/jib.485

Ciani, M., Morales, P., Comitini, F., Tronchoni, J., Canonico, L., Curiel, J.A., Oro, L., Rodrigues, A.J., Gonzalez, R., 2016. Non-conventional Yeast Species for Lowering Ethanol Content of Wines. Front. Microbiol. 7, 642.

Codex Alimentarius (2001) Alinorm 41/0: Revised standard for honey, Alinorm 1, 19–26

Collmer, A., Keen, N.T., 1986. The Role of Pectic Enzymes in Plant Pathogenesis. Annu. Rev. Phytopathol. 24, 383–409. https://doi.org/10.1146/annurev.py.24.090186.002123

Compagno, C., Dashko, S., Piškur, J., 2014. Introduction to Carbon Metabolism in Yeast, in: Molecular Mechanisms in Yeast Carbon Metabolism. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 1–19. https://doi.org/10.1007/978-3-642-55013-3_1

Crane, E., 1991. Honey from honeybees and other insects. Ethol. Ecol. Evol. 3, 100–105. https://doi.org/10.1080/03949370.1991.10721919

Cray, J.A., Bell, A.N.W., Bhaganna, P., Mswaka, A.Y., Timson, D.J., Hallsworth, J.E., 2013. The biology of habitat dominance; can microbes behave as weeds? Microb. Biotechnol. 6, 453–492. https://doi.org/10.1111/1751-7915.12027

da Silva, P.M., Gauche, C., Gonzaga, L.V., Costa, A.C.O., Fett, R., 2016. Honey: Chemical composition, stability and authenticity. Food Chem. 196, 309–323. https://doi.org/10.1016/J.FOODCHEM.2015.09.051

Daniel, H.-M., Prasad, G.S., 2010. The role of culture collections as an interface between providers and users: the example of yeasts. Res. Microbiol. 161, 488–496. https://doi.org/10.1016/J.RESMIC.2010.01.011

Dashko, S., Zhou, N., Tinta, T., Sivilotti, P., Lemut, M.S., Trost, K., Gamero, A., Boekhout, T., Butinar, L., Vrhovsek, U., Piskur, J., 2015. Use of non-conventional yeast improves the wine aroma profile of Ribolla Gialla. J. Ind. Microbiol. Biotechnol. 42, 997–1010. https://doi.org/10.1007/s10295-015-1620-y

Duarte, W.F., Amorim, J.C., Schwan, R.F., 2012. The effects of co-culturing non-Saccharomyces yeasts with S. cerevisiae on the sugar cane spirit (cachaça) fermentation process. Antonie Van Leeuwenhoek 103, 175–94. https://doi.org/10.1007/s10482-012-9798-8

Dzialo, M.C., Park, R., Steensels, J., Lievens, B., Verstrepen, K.J., 2017. Physiology, ecology and industrial applications of aroma formation in yeast. FEMS Microbiol. Rev. 41, S95–S128. https://doi.org/10.1093/femsre/fux031

Ehrlich, F., 1907. Über die Bedingungen der Fuselölbildung und über ihren Zusammenhang mit dem Eiweißaufbau der Hefe. Berichte der Dtsch. Chem. Gesellschaft 40, 1027–1047. https://doi.org/10.1002/cber.190704001156

Fleet, G.H., 2008. Wine yeasts for the future. FEMS Yeast Res. 8, 979–995. https://doi.org/10.1111/j.1567-1364.2008.00427.x

Flores, C.-L., Rodríguez, C., Petit, T., Gancedo, C., 2000. Carbohydrate and energy-yielding metabolism in non-conventional yeasts: Figure 1. FEMS Microbiol. Rev. 24, 507–529. https://doi.org/10.1111/j.1574-6976.2000.tb00553.x

Fromson, D., 2012. Brettanomyces, a Funky Yeast, Makes Flavorful Beers [WWW Document]. New York Times, dec. 27 2012. URL https://www.nytimes.com/2013/01/02/dining/brettanomyces-a-funky-yeast-makes-flavorful-beers.html (accessed 6.2.18).

Gallone, B., Mertens, S., Gordon, J.L., Maere, S., Verstrepen, K.J., Steensels, J., 2018. Origins, evolution, domestication and diversity of Saccharomyces beer yeasts. Curr. Opin. Biotechnol. 49, 148–155. https://doi.org/10.1016/J.COPBIO.2017.08.005

Gamero, A., Quintilla, R., Groenewald, M., Alkema, W., Boekhout, T., Hazelwood, L., 2016. High-throughput screening of a large collection of non-conventional yeasts reveals their potential for aroma formation in food fermentation. Food Microbiol. 60, 147–159. https://doi.org/10.1016/J.FM.2016.07.006

Gomes, T., Barradas, C., Dias, T., Verdial, J., Morais, J.S., Ramalhosa, E., Estevinho, L.M., 2013. Optimization of mead production using Response Surface Methodology. Food Chem. Toxicol. 59, 680–686. https://doi.org/10.1016/J.FCT.2013.06.034

Gonzalez, R., Quirós, M., Morales, P., 2013. Yeast respiration of sugars by non-Saccharomyces yeast species: A promising and barely explored approach to lowering alcohol content of wines. Trends Food Sci. Technol. 29, 55–61. https://doi.org/10.1016/j.tifs.2012.06.015

Hazelwood, L.A., Daran, J.-M., van Maris, A.J.A., Pronk, J.T., Dickinson, J.R., 2008. The Ehrlich pathway for fusel alcohol production: a century of research on Saccharomyces cerevisiae metabolism. Appl. Environ. Microbiol. 74, 2259–66. https://doi.org/10.1128/AEM.02625-07

Hernandez, C., Serrato, J.C., Quicazan, M., 2015. Evaluation of Physicochemical and Sensory Aspects of Mead, Produced by Different Nitrogen Sources and Commercial Yeast.

Chem. Eng. Trans. 43, 1–6. https://doi.org/10.3303/CET1543001

Hernández-Orte, P., Cacho, J.F, Ferreira, V., 2002. Relationship between Varietal Amino Acid Profile of Grapes and Wine Aromatic Composition. Experiments with Model Solutions and Chemometric Study. https://doi.org/10.1021/JF011395O

Hirst, M.B., Richter, C.L., 2016. Review of Aroma Formation through Metabolic Pathways of Saccharomyces cerevisiae in Beverage Fermentations. Am. J. Enol. Vitic. 67, 361–370. https://doi.org/10.5344/ajev.2016.15098

Hittinger, C.T., Rokas, A., Bai, F.-Y., Boekhout, T., Gonçalves, P., Jeffries, T.W., Kominek, J., Lachance, M.-A., Libkind, D., Rosa, C.A., Sampaio, J.P., Kurtzman, C.P., 2015. Genomics and the making of yeast biodiversity. Curr. Opin. Genet. Dev. 35, 100–109. https://doi.org/10.1016/J.GDE.2015.10.008

Holt, S., Mukherjee, V., Lievens, B., Verstrepen, K.J., Thevelein, J.M., 2018. Bioflavoring by non-conventional yeasts in sequential beer fermentations. Food Microbiol. 72, 55–66. https://doi.org/10.1016/J.FM.2017.11.008

Hui, Y.H., 2004. Handbook of Food and Beverage Fermentation Technology. CRC Press.

Jolly, N.P., Varela, C., Pretorius, I.S., 2013. Not your ordinary yeast: non-Saccharomyces yeasts in wine production uncovered. FEMS Yeast Res. 14, 1–23. https://doi.org/10.1111/1567-1364.12111

Janssens, D., Arahal, D.R., Bizet, C., Garay, E., 2010. The role of public biological resource centers in providing a basic infrastructure for microbial research. Res. Microbiol. 161, 422–429. https://doi.org/10.1016/J.RESMIC.2010.03.009

Juan-Borrás, M., Domenech, E., Hellebrandova, M., Escriche, I., 2014. Effect of country origin on physicochemical, sugar and volatile composition of acacia, sunflower and tilia honeys. Food Res. Int. 60, 86–94. https://doi.org/10.1016/J.FOODRES.2013.11.045

Johnson, E.A., Echavarri-Erasun, C., 2011. Yeast Biotechnology, in: The Yeasts. Elsevier, pp. 21–44. https://doi.org/10.1016/B978-0-444-52149-1.00003-3

Jung, S.-T., Rhim, J.-W., Kim, D.-H., 1999. Fermentation Characteristics of Honey Wine by Saccharomyces bayanus, Korean Journal of Food Science and Technology.

Kettmann, M., 2016. America's Mead Renaissance | Wine Enthusiast Magazine [WWW Document]. URL https://www.winemag.com/listicle/americas-mead-renaissance/ (accessed 6.1.18).

Kurtzman, C.P., Fell, J.W., Boekhout, T., 2010. The yeasts: a taxonomic study. Elsevier.

Kurtzman, C.P., Robnett, C.J., Basehoar-Powers, E., 2008. Phylogenetic relationships among species of *Pichia, Issatchenkia* and *Williopsis* determined from multigene sequence analysis, and the proposal of *Barnettozyma* gen. nov., *Lindnera* gen. nov. and *Wickerhamomyces* gen. nov. FEMS Yeast Res. 8, 939–954. https://doi.org/10.1111/j.1567-1364.2008.00419.x

Langkjær, R.B., Cliften, P.F., Johnston, M., Piškur, J., 2003. Yeast genome duplication was followed by asynchronous differentiation of duplicated genes. Nature 421, 848–852. https://doi.org/10.1038/nature01419

Leandro, M.J., Sychrova, H., Prista, C., Loureiro-Dias, M.C., 2011. The osmotolerant fructophilic yeast Zygosaccharomyces rouxii employs two plasma-membrane fructose uptake systems belonging to a new family of yeast sugar transporters. Microbiology 157, 601–608. https://doi.org/10.1099/mic.0.044446-0

Lievens, B., Hallsworth, J.E., Pozo, M.I., Belgacem, Z. Ben, Stevenson, A., Willems, K.A., Jacquemyn, H., 2015. Microbiology of sugar-rich environments: diversity, ecology and system constraints. Environ. Microbiol. 17, 278–298. https://doi.org/10.1111/1462-2920.12570

Liu, W., Zhang, B., Jiang, R., 2017. Improving acetyl-CoA biosynthesis in Saccharomyces cerevisiae via the overexpression of pantothenate kinase and PDH bypass. Biotechnol. Biofuels 10, 41. https://doi.org/10.1186/s13068-017-0726-z

Masoud, W., Poll, L., Jakobsen, M., 2005. Influence of volatile compounds produced by yeasts predominant during processing of Coffea arabica in East Africa on growth and ochratoxin A (OTA) production by Aspergillus ochraceus. Yeast 22, 1133–1142. https://doi.org/10.1002/yea.1304

McGovern, P.E., Zhang, J., Tang, J., Zhang, Z., Hall, G.R., Moreau, R.A., Nuñez, A., Butrym, E.D., Richards, M.P., Wang, C.-S., Cheng, G., Zhao, Z., Wang, C., 2004. Fermented beverages of pre- and proto-historic China. Proc. Natl. Acad. Sci. U. S. A. 101, 17593–8. https://doi.org/10.1073/pnas.0407921102

Mendes-Ferreira, A., Cosme, F., Barbosa, C., Falco, V., Inês, A., Mendes-Faia, A., 2010. Optimization of honey-must preparation and alcoholic fermentation by Saccharomyces cerevisiae for mead production. Int. J. Food Microbiol. 144, 193–198. https://doi.org/10.1016/J.IJFOODMICRO.2010.09.016

Mertens, S., Steensels, J., Saels, V., De Rouck, G., Aerts, G., Verstrepen, K.J., 2015. A large set of newly created interspecific Saccharomyces hybrids increases aromatic diversity in lager beers. Appl. Environ. Microbiol. 81, 8202–14. https://doi.org/10.1128/AEM.02464-15

Moreira, N., Mendes, F., Hogg, T., Vasconcelos, I., 2005. Alcohols, esters and heavy sulphur compounds production by pure and mixed cultures of apiculate wine yeasts. Int. J. Food Microbiol. 103, 285–294. https://doi.org/10.1016/J.IJFOODMICRO.2004.12.029

Moskowitz, H.R., 1970. Ratio scales of sugar sweetness. Percept. Psychophys. 7, 315–320. https://doi.org/10.3758/BF03210175

Naglik, J.R., Challacombe, S.J., Hube, B., 2003. Candida albicans secreted aspartyl proteinases in virulence and pathogenesis. Microbiol. Mol. Biol. Rev. 67, 400–28, table of contents. https://doi.org/10.1128/MMBR.67.3.400-428.2003

Navrátil, M., Šturdík, E., Gemeiner, P., 2001. Batch and continuous mead production with pectate immobilised, ethanol-tolerant yeast. Biotechnol. Lett. 23, 977–982. https://doi.org/10.1023/A:1010571208324

Pereira, A.P., Dias, T., Andrade, J., Ramalhosa, E., Estevinho, L.M., 2009. Mead production: Selection and characterization assays of Saccharomyces cerevisiae strains. Food Chem. Toxicol. 47, 2057–2063. https://doi.org/10.1016/J.FCT.2009.05.028

Pereira, A.P., Mendes-Ferreira, A., Estevinho, L.M., Mendes-Faia, A., 2015. Improvement of mead fermentation by honey-must supplementation. J. Inst. Brew. 121, 405–410. https://doi.org/10.1002/jib.239

Pereira, A.P., Mendes-Ferreira, A., Oliveira, J.M., Estevinho, L.M., Mendes-Faia, A., 2015. Mead production: effect of nitrogen supplementation on growth, fermentation profile and aroma formation by yeasts in mead fermentation. J. Inst. Brew. 121, 122–128. https://doi.org/10.1002/jib.184

Péter, G., Rosa, C. (Eds.), 2006. Biodiversity and Ecophysiology of Yeasts, The Yeast Handbook. Springer-Verlag, Berlin/Heidelberg. https://doi.org/10.1007/3-540-30985-3

Pires, E.J., Teixeira, J.A., Brányik, T., Vicente, A.A., 2014. Yeast: the soul of beer's aroma—a review of flavour-active esters and higher alcohols produced by the brewing yeast. Appl. Microbiol. Biotechnol. 98, 1937–1949. https://doi.org/10.1007/s00253-013-5470-0

Piškur, J., Rozpędowska, E., Polakova, S., Merico, A., Compagno, C., 2006. How did Saccharomyces evolve to become a good brewer? Trends Genet. 22, 183–186. https://doi.org/10.1016/J.TIG.2006.02.002

Pretorius, I.S., 2000. Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. Yeast 16, 675–729.

Pronk, J.T., Steensma, Y.H., Van Dijken, J.P., 1996. Pyruvate Metabolism in *Saccharomyces cerevisiae*. Yeast 12, 1607–1633

Pylro, V.S., Roesch, L.F.W., Ortega, J.M., Amaral, A.M.D., Tótola, M.R., Hirsch, P.R., Rosado, A.S., Góes-Neto, A., Da Costa Da Silva, A.L., Rosa, C.A., Morais, D.K., Andreote, F.D., Duarte, G.F., Melo, I.S.D., Seldin, L., Lambais, M.R., Hungria, M., Peixoto, R.S., Kruger, R.H., Tsai, S.M., Azevedo, V., 2013. Brazilian Microbiome Project: Revealing the Unexplored Microbial Diversity—Challenges and Prospects. Microbial Ecology 67, 237–241. https://doi.org/10.1007/s00248-013-0302-4

Radovic, B.S., Careri, M., Mangia, A., Musci, M., Gerboles, M., Anklam, E., 2001. Contribution of dynamic headspace GC–MS analysis of aroma compounds to authenticity testing of honey. Food Chem. 72, 511–520. https://doi.org/10.1016/S0308-8146(00)00263-6

Ramalhosa, E., Gomes, T., Pereira, A.P., Dias, T., Estevinho, L.M., 2011. Mead Production: Tradition Versus Modernity. Adv. Food Nutr. Res. 63, 101–118. https://doi.org/10.1016/B978-0-12-384927-4.00004-X

Ravasio, D., Carlin, S., Boekhout, T., Groenewald, M., Vrhovsek, U., Walther, A., Wendland, J., 2018. Adding Flavor to Beverages with Non-Conventional Yeasts. Fermentation 4, 15. https://doi.org/10.3390/fermentation4010015

Roldán, A., van Muiswinkel, G.C.J., Lasanta, C., Palacios, V., Caro, I., 2011. Influence of pollen addition on mead elaboration: Physicochemical and sensory characteristics. Food Chem. 126, 574–582. https://doi.org/10.1016/J.FOODCHEM.2010.11.045

Romano, P., Fiore, C., Paraggio, M., Caruso, M., Capece, a., 2003. Function of yeast species and strains in wine flavour. Int. J. Food Microbiol. 86, 169–180. https://doi.org/10.1016/S0168-1605(03)00290-3

Saerens, S.M.G., Verstrepen, K.J., Van Laere, S.D.M., Voet, A.R.D., Van Dijck, P., Delvaux, F.R., Thevelein, J.M., 2006. The Saccharomyces cerevisiae EHT1 and EEB1 genes encode novel enzymes with medium-chain fatty acid ethyl ester synthesis and hydrolysis capacity. J. Biol. Chem. 281, 4446–56. https://doi.org/10.1074/jbc.M512028200

Saerens, S.M.G., Delvaux, F.R., Verstrepen, K.J., Thevelein, J.M., 2010. Production and biological function of volatile esters in *Saccharomyces cerevisiae*. Microb. Biotechnol. 3, 165–177. https://doi.org/10.1111/j.1751-7915.2009.00106.x

Scanes, K.T., Hohrnann, S., Prior, B.A., 2017. Glycerol Production by the Yeast Saccharomyces cerevisiae and its Relevance to Wine: A Review. South African J. Enol. Vitic. 19, 17–24. https://doi.org/10.21548/19-1-2239

Schramm, K., 2003. The compleat meadmaker : home production of honey wine from your first batch to award-winning fruit and herb variations. Brewers Publications.

Snoek, T., Picca Nicolino, M., Van den Bremt, S., Mertens, S., Saels, V., Verplaetse, A., Steensels, J., Verstrepen, K.J., 2015. Large-scale robot-assisted genome shuffling yields industrial Saccharomyces cerevisiae yeasts with increased ethanol tolerance. Biotechnol. Biofuels 8, 32. https://doi.org/10.1186/s13068-015-0216-0

Sprague, G.F., Winans, S.C., 2006. Eukaryotes learn how to count: quorum sensing by yeast. Genes Dev. 20, 1045–9. https://doi.org/10.1101/gad.1432906

Sroka, P., Tuszyński, T., 2007. Changes in organic acid contents during mead wort fermentation. Food Chem. 104, 1250–1257. https://doi.org/10.1016/j.foodchem.2007.01.046

Steensels, J., Verstrepen, K.J., 2014. Taming Wild Yeast: Potential of Conventional and Nonconventional Yeasts in Industrial Fermentations. Annu. Rev. Microbiol. 68, 61–80. https://doi.org/10.1146/annurev-micro-091213-113025

Steensels, J., Meersman, E., Snoek, T., Saels, V., Verstrepen, K. J., 2014. Large-scale selection and breeding to generate industrial yeasts with superior aroma production. Applied and environmental microbiology, AEM-02235.

Stewart, G.G., Russell, I., 1986. One hundred years of yeast research and development in the brewing industry. J. Inst. Brew. 92, 537–558. https://doi.org/10.1002/j.2050-0416.1986.tb04453.x

Styger, G., Prior, B., Bauer, F.F., 2011. Wine flavor and aroma. J. Ind. Microbiol. Biotechnol. 38, 1145–1159. https://doi.org/10.1007/s10295-011-1018-4

Suwannarangsee, S., Oh, D.-B., Seo, J.-W., Kim, C.H., Rhee, S.K., Kang, H.A., Chulalaksananukul, W., Kwon, O., 2010. Characterization of alcohol dehydrogenase 1 of the thermotolerant methylotrophic yeast Hansenula polymorpha. Appl. Microbiol. Biotechnol. 88, 497–507. https://doi.org/10.1007/s00253-010-2752-7

Tronchoni, J., Medina, V., Guillamón, J., Querol, A., Pérez-Torrado, R., 2014. Transcriptomics of cryophilic Saccharomyces kudriavzevii reveals the key role of gene translation efficiency in cold stress adaptations. BMC Genomics 15, 432. https://doi.org/10.1186/1471-2164-15-432

Ukpabi, U.J., 2006. Quality evaluation of meads produced with cassava(Manihot esculenta) floral honey under farm conditions in Nigeria. Trop. Subtrop. Agroecosystems 6.
Van Breda, V., Jolly, N., van Wyk, J., 2013. Characterisation of commercial and natural Torulaspora delbrueckii wine yeast strains. Int. J. Food Microbiol. 163, 80–88. https://doi.org/10.1016/j.ijfoodmicro.2013.02.011

Verstrepen, K.J., Van Laere, S.D.M., Vanderhaegen, B.M.P., Derdelinckx, G., Dufour, J.-P., Pretorius, I.S., Winderickx, J., Thevelein, J.M., Delvaux, F.R., 2003. Expression levels of the yeast alcohol acetyltransferase genes ATF1, Lg-ATF1, and ATF2 control the formation of a broad range of volatile esters. Appl. Environ. Microbiol. 69, 5228–37.

Viana, F., Gil, J. V., Genovés, S., Vallés, S., Manzanares, P., 2008. Rational selection of non-Saccharomyces wine yeasts for mixed starters based on ester formation and enological traits. Food Microbiol. 25, 778–785. https://doi.org/10.1016/j.fm.2008.04.015

Vidrih, R., Hribar, J., 2007. Studies on the sensory properties of mead and the formation of aroma compounds related to the type of honey. Acta Aliment. 36, 151–162. https://doi.org/10.1556/AAlim.36.2007.2.2

Wagner, J.M., Alper, H.S., 2016. Synthetic biology and molecular genetics in non-conventional yeasts: Current tools and future advances. Fungal Genet. Biol. 89, 126–136. https://doi.org/10.1016/J.FGB.2015.12.001

White, J., Doner, L., 1980. Honey composition and properties, Beekeeping in the United States Agriculture Handbook. US Department of Agriculture. Retrieved September 21, 2018 from https://beesource.com/resources/usda/honey-composition-and-properties/

World Federation for Culture Collections, n.d. Culture Collections Information Worldwide. Retrieved May 23, 2018 from http://www.wfcc.info/ccinfo/index.php/collection/by_id/1083/

Zinjarde, S.S., 2014. Food-related applications of *Yarrowia lipolytica*. Food Chem. 152, 1–10. https://doi.org/10.1016/J.FOODCHEM.2013.11.117

5 METHOD DEVELOPMENT FOR GAS CHROMATOGRAPHY- MASS SPECTROMETRY

5.1 Comparison of solvent extraction versus solid phase micro-extraction and automatization of data analysis

One underlying objective of the current research project was to establish a high-throughput workflow for chromatography analysis, enabling the screening of a high number of yeasts from the culture collection for production of volatile organic compounds (VOC's) under various experimental conditions, in a short amount of time.

This chapter briefly discusses some of the work that was undertaken to develop an in-house protocol for gas chromatography – mass spectrometry and data analysis, but is not intended for publication.

Under current conditions the laboratory is equipped with a Shimadzu GCMS QP2010-SE combined with an AOC-20i auto-injector which can inject a queue of liquid samples through a needle, but not of performing SPME extractions. Therefore, a workflow that includes liquid samples can potentially run non-stop; with a run time of 50 min per sample, in theory up to 201 samples could be analyzed on a weekly basis. While using the manual SPME procedure one is limited to the working hours of the GC operant; assuming a workweek of 40 h and a run time of 50 min per sample, at most 48 samples a week could be analyzed. In conclusion, using a solvent extraction would allow 4x as much samples to be analyzed within the same period of time.

However, there are potential drawbacks to consider when developing a solvent extraction methodology: (1) the solvent could increase the cost per injection, if an expensive solvent or high volumes are required; (2) solvent injections might shorten the lifetime of a column or reduce MS sensitivity; (3) a solvent might be inefficient at extracting the compounds of interest; (4) the solvent extraction procedure might be time consuming, therefore undoing its potential advantage in terms of throughput; (5) a solvent might be damaging to the environment or induce workplace hazards.

In practice, drawbacks (1) and (2) prohibit the use of certain solvents that are incompatible for use in combination with the desired column (OV Carbowax) or GC-MS in general. For example, water is an unsuitable solvent for injections in GC-MS systems as it is damaging to most common polar coatings and its high vapour volume reduces the vacuum in the MS system (Aeppli et al., 2008). These considerations exclude the possibility of directly injecting an aqueous sample.

Drawbacks (3) and (4) should be overcome by optimizing an extracting methodology for recovery of target metabolites and reducing the time requirements. A literature review showed that the most common solvents used for the extraction of volatiles from fermented beverages (in most cases, wine) are dichloromethane, hexane and diethyl ether (e.g. Romano, 2009; Mamede and Pastore, 2006). After verification that these solvents are compatible for use with the intended column, it was chosen to proceed towards testing extractions using dichloromethane and a 1:1 (v/v) mixture of hexane and diethyl ether.

Protocols requiring high solvent volumes (e.g. 1 l of medium and 100 ml of solvent) were not taken into consideration because those would be too costly to process large amounts of samples (see drawback 1). Also, protocols that require an evaporation step were discarded; while concentration benefits the recovery of minor VOCs, this would make the procedure too time consuming (see drawback 4).

The first protocol that was considered for the extraction used only 0.2 ml of solvent (Ortega et al., 2001). However, in practice this low volume was insufficient to achieve a good phase separation as emulsification effects seemed to occur. Several remedies aiming to resolve this issue (addition of salt, cooling down on ice and centrifugation) were attempted, but to no avail.

Therefore, a second protocol was considered using a higher solvent volume (two extractions with 2 ml of solvent) (Romano et al., 2009). This lead to an improved phase separation and acceptable reproducibility.

An example of chromatograms produced by extraction performed, by solvent extractions and SPME extraction, on fermented culture medium is shown in Figure 1. It can readily be observed that the SPME chromatogram has a higher number of peaks, that also have larger peak areas than solvent extraction chromatograms; however, conclusions cannot be drawn based on the chromatogram alone because peaks do not necessarily correspond to compounds of interest. For example, diethyl ether – hexane seems to extract some compounds that are not extracted by SPME. However, closer examination identified these compounds as butylated hydroxytoluene (RT 17.309) and hexadecane (RT 12.597) which are likely contaminations of the solvents (HPLC grade solvents were used) rather than compounds of interest.

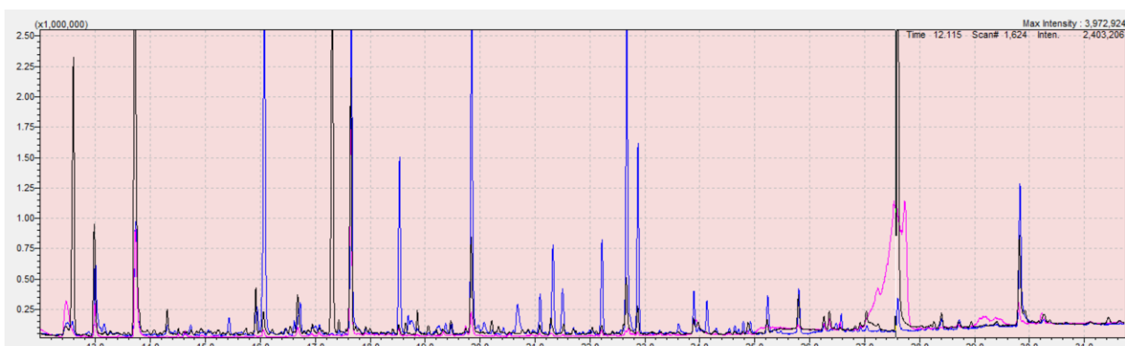

Figure 1. Center zone of chromatograms obtained with dichloromethane (pink), diether-hexane (black) solvent extractions and DVB/CAR/PDMS SPME (blue)

When focusing on actual compounds of interest, SPME extraction showed a superior range of action (Table 1) particularly regarding ethyl esters which were not detected in either of the solvent extracts. This might be because the fiber has been coated with three separate materials (DVB/CAR/PDMS), of different polarities, selected to extract a wide range of components. On the other hand, diethyl ether-hexane does achieve a similar or even superior extraction for all other compounds. Dichloromethane extracted the lowest number of targeted compounds, in particular esters.

Table 1. Qualitative comparison of extracted metabolites of interest between SPME, diether-hexane and dichloromethane extraction

Compound (RT)	SPME	Diethyl ether-hexane	Dichloromethane
Octanoic acid, ethyl ester (8.879)	X		
Propanoic acid, 2-methyl- (11.819)	X	X	X
Dodecanoic acid, ethyl ester (16.465)	X		
Phenylethyl alcohol (17.644)	X	X	X
Isopropyl palmitate (22.054)	X	X	

Compound (RT)	SPME	Diethyl ether-hexane	Dichloromethane
Octadecanoic acid, ethyl ester (24.705)	X		
Benzeneacetic acid (26.375)	X	X	X
Benzyl benzoate (26.827)	X	X	

Based on the results and considerations described above, it was chosen to proceed with SPME extractions for three reasons:

1) Failure to extract ethyl esters with solvent diethyl ether-hexane. Ethyl esters are compounds of primary interest when doing research on aroma production by non-conventional yeasts. For other applications, this restriction might not apply.

2) With equipment currently available in the lab (magnetic stirring plates, separation funnels) sample preparation time still amounted to approximately 30 min per sample, which is hardly superior to the manual SPME procedure. To process enough samples to achieve the maximal weekly throughput of 200 samples a week the lab would need to invest in this equipment.

3) Given the lack of specific advantages of using a solvent extraction, SPME remains preferable because it is safer in terms of environmental and workplace hazards as well.

It is possible that further optimization of the solvent extraction protocol, potentially including concentration of the sample by evaporation or selection of other solvents, would improve the extraction of ethyl esters.

5.2 Development of a protocol for data analysis

Most fermented foods contain complex mixtures of volatile organic compounds that are hard to analyze by gas chromatography, as they contain both the volatiles produced by micro-organisms during fermentation and those coming from the original substrate (in the context of this research, honey). In practice, this means that there is a high number of peaks, some of which may overlap; and that chromatograms are noisy, making it harder to reliable identify some of the compounds.

A correct approach to chromatographic data analysis should include processes for peak picking, peak alignment and identification (Sumner et al., 2005). Whereas peak picking and alignment can be performed efficiently with Shimadzu's proprietary software, its identification process is lacking.

Peak identification of GC-MS data should be based on both mass spectral data (similarity of a peak's spectrum with a spectrum of a pure compound in a database) and Kovat's retention index (based on the retention time of compounds relative to a standard, which can be compared between labs). The Shimadzu software does not provide a way to take both types of data into account simultaneously.

To address this shortcoming, an algorithm was developed that starts where the alignment procedure of Shimadzu's software ends, to perform identifications. This algorithm has three benefits versus a manual continuation of the data processing: it is significantly faster, it considers all available data and leaves no space for human errors.

The software joins together peak areas from individual chromatograms and writes them into a large table. Then it reads the database search files and lists all possible identities for each compound and their similarity (SI). For all possible identities, it automatically looks up retention indices (RIs) in the NIST Chemistry WebBook (https://webbook.nist.gov/chemistry). Only RI values that match the specified column, or similar ones are considered (for example, DB-WAX and OV-Carbowax are essentially the same columns and are expected to have comparable retention indexes).

For each possible identity, it is verified whether the experimental RI and literature RI are close enough (threshold is 50 by default). The closest match to a literature is selected and written to the output file, along with its source. The final identification can have several possible outcomes:

- no MS match, meaning that no compound with a SI of 90% or greater was found in the database (the user can adjust this value);
- no RI match, meaning that all the available options based on mass spectra have too divergent RI values from the literature to be considered a possible match;
- multiple matches, meaning that more than one compound has a sufficiently high SI and close enough RI. In this case, the compound with the highest SI is chosen as the most likely identity but the user is alerted of other possibilities;
- a positive identification, meaning that (only) one compound matches both the SI and RI requirements.

The most likely identities are then written into the table with peak areas. The user can also read back the decision process of the algorithm, per compound that was listed as a possible identity, and adjust if necessary.

The source code of this algorithm can be reviewed is available on request. It could be further improved by automatic reading of the NIST database file instead of the online version, or by including automatic statistical analyses and data visualizations.

5.3 Materials and methods

Solvent extractions

Two methods for solvent extraction were tried out, as described previously (Romano et al., 2009) with modifications. Ten ml of fermented medium was extracted twice with 2 ml of diethyl ether – hexane (1:1 (v/v) mixture) (method 1) or dichloromethane (method 2) in a 25 ml Erlenmeyer flask for 15 min while stirred with a magnetic bar. Samples were poured into a separation funnel and after phase separation occurred the organic phase was collected, dried with sodium sulphate and 1 µl was injected into the GC-MS using an autosampler.

Solid phase microextractions

Volatile compounds were extracted using a manual headspace-solid phase microextraction procedure (HS–SPME) with a divinylbenzene/carboxen/polydimethylsiloxane 50/30 µm SPME fiber (Supelco Co., Bellefonte, PA, USA). Two ml of liquid sample was mixed with 0.5 g of sodium chloride (to improve extraction efficiency) and placed in a 10 ml hermetically sealed vial. After equilibration at 60 °C for 15 min, the volatile compounds were extracted at 60 °C 30 min. Desorption time on the column was 3 min.

Gas chromatography – mass spectrometry

A GCMS-QP2010 (Shimadzu), equipped with an OV Carbowax column (30 m × 0.25 mm × 0.25 µm) was used for GC/MS analysis. The oven temperature was maintained at 50 °C for 5 min, then raised to 190 °C at 3 °C/min and maintained at 190 °C for 10 min. The injector and detector were maintained at 230 and 240 °C, respectively. The He carrier gas was maintained at a flow rate of 1.2 ml/min. Volatile compounds were identified by comparison of their mass spectra to the NIST 11 database.

5.4 References

Aeppli, C., Berg, M., Hofstetter, T.B., Kipfer, R., Schwarzenbach, R.P., 2008. Simultaneous quantification of polar and non-polar volatile organic compounds in water samples by direct aqueous injection-gas chromatography/mass spectrometry. J. Chromatogr. A 1181, 116–124. https://doi.org/10.1016/J.CHROMA.2007.12.043

Ortega, C., López, R., Cacho, J., Ferreira, V., 2001. Fast analysis of important wine volatile compounds: Development and validation of a new method based on gas chromatographic–flame ionisation detection analysis of dichloromethane microextracts. J. Chromatogr. A 923, 205–214. https://doi.org/10.1016/S0021-9673(01)00972-4

Romano, A., Perello, M.C., Lonvaud-Funel, A., Sicard, G., de Revel, G., 2009. Sensory and analytical re-evaluation of "Brett character." Food Chem. 114, 15–19. https://doi.org/10.1016/j.foodchem.2008.09.006

Sumner, L.W., Urbanczyk-Wochniak, E., Broeckling, C.D., 2005. Metabolomics Data Analysis, Visualization, and Integration, in: Plant Bioinformatics. Humana Press, Totowa, NJ, pp. 409–436. https://doi.org/10.1007/978-1-59745-535-0_20

Article 1: SCREENING OF VOLATILE ORGANIC COMPOUNDS PRODUCED BY YEASTS FROM A BRAZILIAN CULTURE COLLECTION (CCMA) AND SELECTION OF STRAINS WITH POTENTIAL AS BIO-FLAVORING AGENTS

This article has been formatted according to the guidelines of the journal *Food microbiology*.

Abstract

The Culture Collection of Agricultural Microbiology (CCMA) is a public service culture collection of micro-organisms that were isolated from various agricultural niches and fermentation processes in Brazil. This study aimed to assess production of aroma compounds by yeasts from this collection and their potential to contribute to flavour development in alcoholic beverages. Production of volatile organic compounds by selected strains (n=63) during culture medium fermentations was assessed by gas chromatography – mass spectrometry. Several relevant aroma compounds were detected of which production was strain-dependent. Based on Pearson correlation between aroma profiles, yeasts were grouped into three main clusters with distinct characteristics. Yeasts with attractive aroma profiles (n=19) were selected for small-scale mead fermentations. While several yeasts did not grow well on honey must, seven strains were able to produce a sweet, low-alcoholic mead (45-168 g/l of residual sugar, 3.8-6.5% ethanol v/v). A screening of volatile organic compounds produced in these meads revealed that *S. cerevisiae* CCMA 1523 produced a high amount of medium chain fatty acid ethyl esters but low amounts of phenethyl acetate. Several yeast strains, including *Torulaspora delbrueckii* CCMA 1524, *Kluyveromyces lactis* CCMA 1518 and *Pichia caribbica* CCMA 0198 produced over ten-fold higher amounts of this compound. These results highlight the potential of non-conventional yeast strains to contribute to flavour development during mead fermentations.

Keywords: yeast culture collection, non-conventional yeasts, aroma compounds, bio-flavoring, mead

1 Introduction

Public service microbial culture collections provide essential services to the scientific community and society, including the preservation and distribution of microbial strains and their associated information. They facilitate independent verification of published results, enable research designs that incorporate a greater diversity of strains and are instrumental in efforts to select optimal strains for biotechnological applications.

In the area of food fermentation research, a wide range of products from various geographic origins has been studied over the last decades. Although strains that were isolated in these studies may possess properties that are of interest for both researchers and the food industry, many of them have not been deposited in public service microbial culture collections and are therefore presumed lost (Daniel and Prasad, 2010).

In Brazil, the Laboratory of Microbial Physiology and Genetics of the Universidade Federal de Lavras (UFLA) has carried out research projects on the microbiology of fermented foods (including coffee, cocoa and indigenous fermented beverages), silage and soil for over 20 years. Recognizing the value of micro-organisms that have been isolated from these often understudied niches, more than 2000 strains of bacteria and yeasts have been made available to the public through the Culture Collection of Agricultural Microbiology (CCMA), which is registered under number 1083 at the World Federation of Culture Collections since 2014 (World Federation for Culture collections, n.d.).

A potential area of application for yeasts in the CCMA collection is bio-flavoring, since it has been demonstrated that strains of several non-*Saccharomyces* species produce desirable aroma-active compounds in significantly higher concentrations than commercial strains of *Saccharomyces cerevisiae* (Ravasio et al., 2018; Steensels and Verstrepen, 2014). These yeasts have also received interest from the brewing industry, as they can contribute additional complexity to the sensory profile of fermented beverages. Mead (honey wine) may be an attractive product for application of non-conventional yeast starter cultures, since compounds of interest that were found in previous aroma screening studies (Gamero et al., 2016; Holt et al., 2018) include esters that represent floral or fruity aromas. These aromas may be a good complement to the aroma profile of meads as they are associated with superior honey quality (Anupama et al., 2003).

While several studies have focussed on the selection of strains of *Saccharomyces cerevisiae* or *Saccharomyces bayanus* and optimization of fermentation parameters during mead production (Jung et al., 1999; Mendes-Ferreira et al., 2010; Pereira et al., 2009), so far

little is known about the application of non-conventional yeasts in the production of this beverage.

In this work, the variety in aroma production among yeasts from the CCMA collection as well as their suitability for bio-flavoring of alcoholic beverages are assessed. In a first experiment, a screening of aroma production on culture media is performed. This provides a general overview of aroma production by yeasts in this collection and its distribution among genera and species. Yeasts that produce high amounts of aroma compounds that are relevant for bio-flavoring are selected for a second experiment that involves small scale mead fermentations. The objective is to select strains that have potential for application as bio-flavoring agents in mead and other alcoholic fermentations.

2 Materials and methods

2.1 Strains

All yeast isolates characterized in this study (n= 63) come from the of the CCMA collection. The yeasts have been isolated from various agricultural substrates including indigenous beverages, coffee, cocoa, sugar cane, silage, soil. A complete list can be found in Appendix 1.

2.2 Storage and re-activation

Yeast stocks were stored at -80 °C in 20% (w/v) glycerol. For reactivation, a loop of the stock material was streaked on an YEPG agar plate (1% (w/v) yeast extract, 2% soy peptone, 2% glucose, 2% agar) and incubated at 28 °C for at least 16h, depending on the growth of the yeast.

2.3 Culture medium fermentations

Aroma production was evaluated by lab-scale fermentations of culture medium. Pre-cultures were prepared by inoculating an Eppendorf tube with 1 mL of YEPG broth (YEPG without agar) with a loop of cell material from a single colony and incubated at 28 °C for 24 h. Fifty ml tubes were filled with 45 ml of modified YEPG (0.5% (w/v) yeast extract, 0.5% soy peptone, 4% glucose) (Gamero et al., 2016). These were inoculated with 1% of pre-culture and incubated at 25 °C for 72 hours, in well-closed tubes without agitation. Cultures were centrifuged at 9000 rpm, 4 °C for 10 min and supernatants were frozen at -18 °C before analysis of their volatile composition.

2.4 Honey must fermentations

Honey produced by *Apis mellifera* from *Myracrodruon urundeuva*, locally known as aroeira honey (Bastos et al., 2016), was obtained from a beekeeper in Taiobeiras, Minas Gerais, Brazil. Meads were prepared in a similar way as described by Sroka and Tuszynski (2017), with modifications. The honey was mixed with distillate water in a 1:3 (v/v) ratio. The must was supplemented with 0.45 g/l of diammonium phosphate to prevent nitrogen limitation. No salts or vitamins were added, as benefits ascribed to these supplements lack empirical support (Pereira et al., 2015). The must was pasteurized in a water bath at 60 °C for 25 min. Pre-cultures were made by inoculating re-activated yeasts of selected strains from a single colony in the honey must and incubating for 24 hours at 28 °C. Fifty ml tubes were filled with 45 ml of honey must and inoculated with 0.5% of the pre-culture. The tubes were incubated at 25 °C for 28 days in well-closed tubes without agitation. Samples were taken after 14 and 28 days which were frozen at -18 °C before analysis of their volatile composition, sugar and ethanol content. Fermentations were carried out in duplicate.

2.5 Solid phase micro-extractions

Volatile compounds were extracted as described by Ribeiro et al. (2017) with minor modifications, using a manual headspace-solid phase micro-extraction procedure (HS–SPME) with a divinylbenzene/carboxen/polydimethylsiloxane 50/30 µm SPME fiber (Supelco Co., Bellefonte, PA, USA). Two ml of liquid sample was mixed with 0.5 g of sodium chloride, to improve extraction efficiency (Ducki et al., 2008), and placed in a 15 ml hermetically sealed vial. After equilibration at 60 °C for 15 min, the volatile compounds were extracted at 60 °C 30 min. Desorption time on the column was 3 min.

2.6 Gas chromatography – mass spectrometry

Samples were analyzed by gas chromatography – mass spectrometry (GC-MS) to determine their volatile compound composition. Operating conditions were as described by Ribeiro et al. (2017). Compound identification was based on comparison of mass spectra to the NIST 11 library and comparison of retention index based on an alkane series to data reported in the literature, as described by Bressani et al. (2018).

2.7 High performance liquid chromatography

Samples were analyzed by high performance liquid chromatography to determine the concentration of residual sugars (glucose, fructose) and ethanol. They were prepared by

centrifuging twice at 9000 rpm, 4 °C for 10 min and filtering the second supernatant with a 0.22 µm nitrocellulose membrane. HPLC operating conditions, compound identification and quantification were as described by Evangelista et al. (2014) with minor modifications. A Shimadzu liquid chromatography system (Shimadzu Corp., Japan) equipped with a dual detection system consisting of a UV–Vis detector (SPD 10Ai) and a refractive index detector (RID-10Ai) is used. A Shimadzu ion exclusion column, Shim-pack SCR-101 H (7.9 mm x 30 cm) was operated at 30 °C. A perchloric acid solution with pH 2.11 was used as the eluent at a flow rate of 0.6 ml/min. Compounds were detected via RID.

2.8 Data analysis

GC-MS data was analyzed using Shimadzu's proprietary software. Heatmaps were produced using the web-based tool Morpheus (https://software.broadinstitute.org/morpheus) using z-values as input and clustering based on Pearson correlation.

3 Results and discussion

3.1 Culture medium fermentations

The metabolic fingerprint of 63 yeast isolates from the CCMA collection has been determined in lab-scale fermentations on culture medium. The objective was to select yeasts with a variety of flavour profiles, characterized by the production of metabolites that may contribute to a pleasant aroma, for application in the production of alcoholic beverages such as mead.

Across samples, 39 volatile organic compounds (VOC's) were identified: 18 esters, 9 fatty acids, 6 acids, 5 alcohols, and 1 aldehyde. Results of the screening are presented in a heatmap (Figure 1). In this figure, red squares indicate a production above the average for yeasts in this screening of a certain aroma compound, whereas blue squares indicate below average production. Compounds that were not detected for a given yeast strain are marked with a grey square.

In order to analyse the distribution of metabolite profiles among genera and species, the strains were clustered based on Pearson correlation. This resulted in three main groups. The first cluster (labelled C1) contains most strains that are high producers of organic acids of various chain lengths as well as 2,3-butanediol. Strains that are high producers of fatty acid ethyl esters are mostly situated in the second cluster (C2). The third cluster (C3) contains

strains that are high producers of esters of aromatic alcohols as well as higher alcohols (isoamyl alcohol, phenethyl alcohol and methionol).

In this study, no clear-cut separation of species or genera based could be observed on the basis of their metabolite profiles. For example, strains of *Pichia* spp., *Candida* spp. and *S. cerevisiae* can be found among all three clusters. This may be a result of habitat adaptations as individual strains were isolated from substrates as diverse as coffee, cocoa, Amerindian beverages, silage and soil.

Previous studies on aroma production by yeasts also observed significant intraspecies variation (Gamero et al., 2016). A small number of mutations may have a significant impact on the expression of the relevant metabolic pathways, and such mutations may arise from adaptations to specific habitats. Steensels et al. (2014) found a large variation in aroma profiles among wild-type and domesticated strains of *S. cerevisiae.* Since *Pichia* and *Candida* are the best represented genera in this study (with 19 and 21 isolates, respectively), a broad comparison can be made between these two groups of strains. In Figure 1, most *Pichia* isolates are concentrated at the bottom of the heat map (in clusters 2 and 3) whereas most *Candida* isolates are located at the top (in clusters 1 and 2). *Candida* strains were more often producers of 2,3-butanediol and on average produced higher amounts of fatty acids, whereas *Pichia* strains where higher producers of higher alcohols and derived esters. The groups can be distinguished with an ANOVA test based on superior production of phenethyl alcohol ($P < 0.02$) and phenethyl acetate ($P < 0.02$) by *Pichia* strains and heptanoic acid ($P < 0.01$) by *Candida* strains. There are, however, exceptions to this rule; for example, both *Pichia fermentans* CCMA 0195 and *Pichia anomala* CCMA 0193 had below average production of phenethyl alcohol and phenethyl acetate.

3.2 Selection of strains for mead fermentations

Yeast strains with interesting metabolite profiles were selected for a follow-up experiment involving mead production. The challenge when selecting yeasts for specific alcoholic fermentations is to find strains that have an adequate fermentation performance, produce VOC’s that impart desirable aroma’s in the right quantity (enough to be perceived, but not overwhelming) and do not produce high quantities of compounds with undesirable aromas.

Several of the compounds that were produced during the culture medium fermentations are known to be aroma-active. Ethyl esters (such as ethyl hexanoate) are associated with various types of fruity odors whereas aromatic alcohols and derived esters

that were produced (phenethyl alcohol, phenethyl acetate, phenethyl propanoate, benzyl alcohol and benzyl benzoate) represent floral, rose, honey and/or balsamic odors. Also, organic acids with cheesy and/or sweaty odors (propanoic acid, 2-methylpropanoic acid, valeric acid, butanoic, hexanoic, heptanoic and nonanoic acid) were found, alongside the higher alcohol methionol which is associated with a sulfurous odor (Garg et al., 2018).
Increasing fruity and floral aromas has repeatedly been stated as an objective of the application of non-conventional yeasts in alcoholic fermentations (Basso et al., 2016; Gutiérrez et al., 2018). In the context of this study, floral and fruity notes are also considered desirable since they are associated with superior honey quality (Anupama et al., 2003) and may therefore enhance the honey aroma of the mead beverage. Cheese and lactic odors, which may be imparted by some of the organic and fatty acids that were encountered in this study, were deemed unpleasant during a sensory evaluation of mead (Castro-Vázquez et al., 2009).

Evaluating the results of the VOC screening, strains that have an above average production of the desirable aromas are mostly present in C3 of the heatmap in Figure 1. This section contains several yeast species that have previously been described as high producers of desirable aroma compounds during wine fermentations, including *Torulaspora delbrueckii* (Azzolini et al., 2015; van Breda et al., 2013) and *Hansienaspora opuntiae* (Oliveira Assis et al., 2014). Also, the strain *Pichia caribbica* CCMA 0198 which is present in this cluster has been used in previous studies in our lab and resulted in superior quality spirits (Amorim et al., 2016). Strains with closely related aroma production according to their position in this cluster may therefore also produce aroma compounds in quantities that are relevant to alcoholic fermentations.

A list of selected strains (n = 19) to be considered as starter cultures for mead production is show in Appendix 2. Apart from the flavour, potential pathogenicity was also considered when selecting the candidate starter cultures. Selected strains originate from all groups of substrates that were included in this study (soil, silage, cocoa, coffee and indigenous beverages) indicating that yeasts of non-food origins may also produce attractive aroma profiles.

3.3 Fermentation of honey must: fermentation performance

Lab-scale fermentations of honey must were carried out to determine the fermentation performance of selected yeasts. Among the nineteen initially selected strains, five strains did not grow on honey must while another seven strains reached less than 2% of alcohol by

volume after two weeks and were excluded from consideration as potential starter cultures (Appendix 3).

The reason several of the yeasts performed poorly might be the osmotic pressure, resulting from the high initial concentration of sugars, or a low ethanol tolerance. Honey is also known to possess antifungal properties, against *Candida albicans*, owing to the presence of glucose oxidase which produces low amounts of hydrogen peroxide (Irish et al., 2006). This enzyme was shown to remain active upon dilution of honey and is therefore potentially relevant to mead fermentations (Bang et al., 2003). Furthermore, terpenes that are present in honey may have antifungal activity (Manyi-Loh et al., 2011). A previous screening of *Saccharomyces* spp. wine strains as mead starter cultures also reported that a majority of strains did not grow on honey must (Caridi et al., 1999).

The remaining seven yeasts produced sweet, low-alcoholic meads with alcohol concentrations between 3.8 and 6.5% v/v; residual sugar concentrations were between 46 and 168 g/l (Figure 2). Residual sugar is not necessarily undesirable in meads, depending on the chosen brewing style; commercial meads have been reported that contain between 25 and 278 g/l of residual sugars (Steinkraus and Morse, 1973).

The initial honey must contained 168.6 g/l of fructose and 145.1 g/l of glucose. After 4 weeks of fermentation, *Saccharomyces cerevisiae* CCMA 1523 had consumed 92% of glucose and 79% of fructose. Non-*Saccharomyces* yeast strains left a significantly higher amount of residual sugars than *S. cerevisiae*. The amount of residual sugar in meads that are produced with these strains may be controlled by adding less honey to the initial must or by using them as a co-starter culture alongside a strain that consumes more sugars.

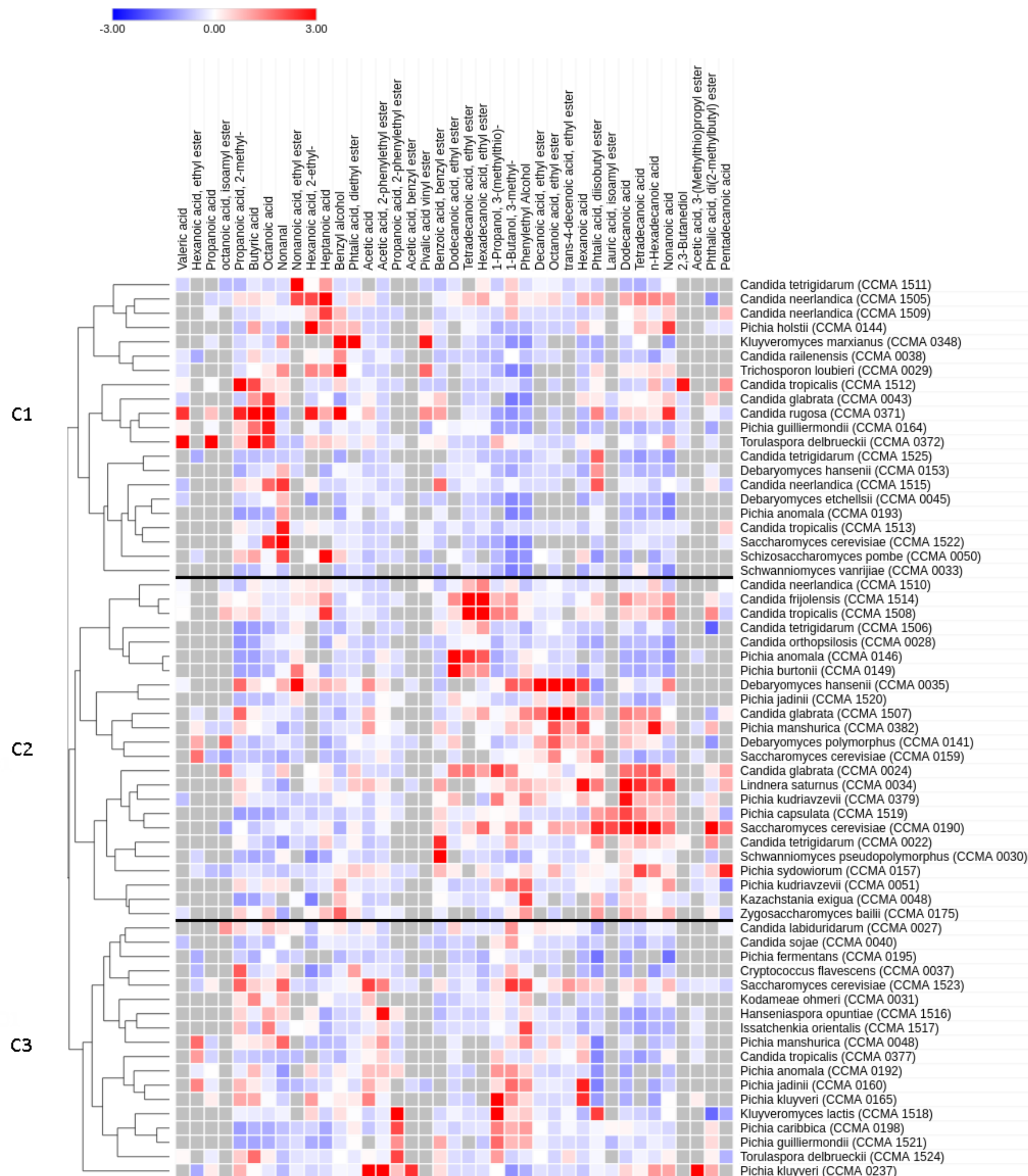

Figure 1. Heatmap of volatile organic compounds produced by yeasts from the CCMA during culture medium fermentations. Numbers are z-values of chromatographic peak areas and yeasts are clustered by Pearson correlation; main clusters are labelled C1-C3. Red squares indicate above average production, blue squares below average, grey squares indicate undetected compounds.

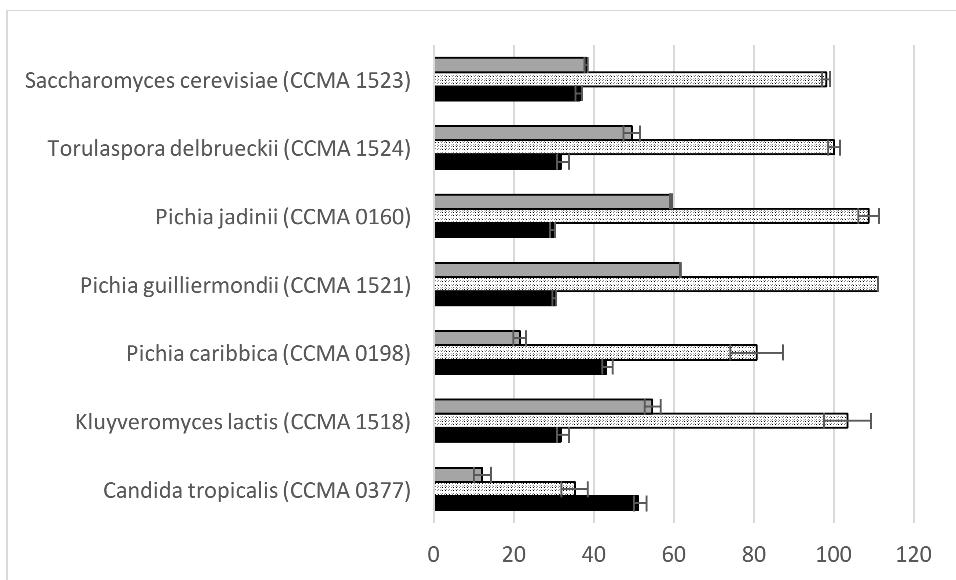

Figure 2. Residual concentrations (in g l^{-1}) of glucose (gray bars), fructose (dotted bars) and production (in g l^{-1}) of ethanol (black bars) in mead after four weeks of fermentation by selected yeast strains.

3.4 Fermentation of honey must: aroma production

Production of volatile organic compounds (VOC's) during lab-scale mead fermentations was determined after four weeks of fermentation. The results are shown in Appendix 4. A total of 49 compounds was identified of which 31 were also present in the must. The remaining compounds are presumed to be of microbial origin. A number of compounds (such as acetic acid and phenethyl alcohol) occur in must but also increase in concentration during the fermentation.

Several compounds of microbial origin are aroma-active, including fatty acid ethyl esters, in the range C8 (ethyl octanoate) to C18 (ethyl octadecanoate); esters of this type are associated with fruity and/ or waxy aromas. The aromatic alcohols benzyl alcohol and phenethyl alcohol (which is also present in the must, but increases in concentration during fermentation) and phenethyl acetate are associated with floral aromas. γ-n-decalactone, which has a peachy aroma, was present in mead but not detected in the screening of culture medium fermentations. As compound concentrations were not quantified in this study, they cannot be discussed in relation to their odor detection threshold. However, peak areas of each compound can be compared between samples.

Peak areas of several fatty acid ethyl esters produced during mead fermentation are shown in Figure 3a. The relative amounts of fatty acid ethyl esters that are produced by each yeast suggests a variation in substrate specificity of the intracellular ester synthases that they possess. For example, *Pichia jadinii* produces a relatively high amount of ethyl octanoate but is among the lowest producers for all other esters that are included in this comparison, suggesting that its ester synthases have a relativity high affinity for octanoyl-CoA as a precursor while those of *S. cerevisiae* would be expected to have a higher affinity for decanoyl-CoA. It should be noted that medium- and long chain fatty acid esters (C8 and higher) do not easily migrate through the plasma membrane (Nykänen et al., 1977). As they are mostly located inside the cell, they might only be released upon cell death (Saerens et al., 2010). The practical relevance of yeasts possessing ester synthases with different substrate affinities is that it offers a potential way to influence the ratio of aroma compounds in fermented foods.

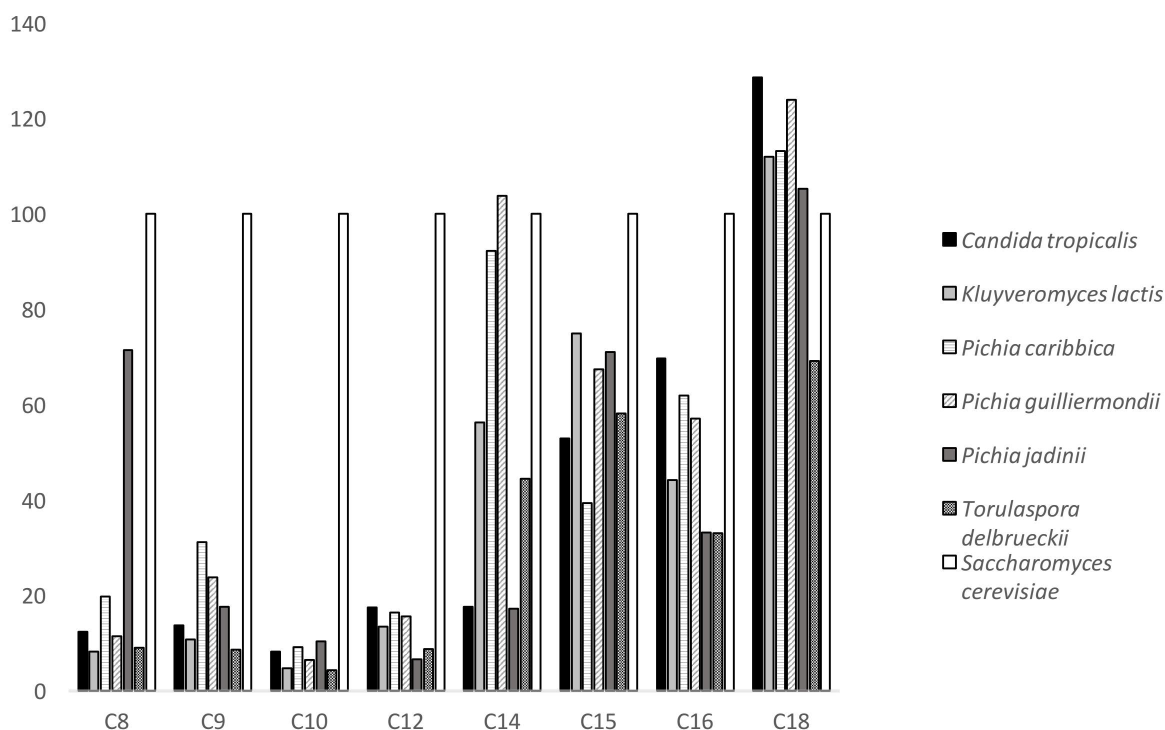

Figure 3a. Normalized peak area of fatty acid ethyl esters (relative to *Saccharomyces cerevisiae* = 100), grouped by fatty acid chain size, after 4 weeks of mead fermentation by selected yeast strains. Standard deviations are approximately 15% of mean.

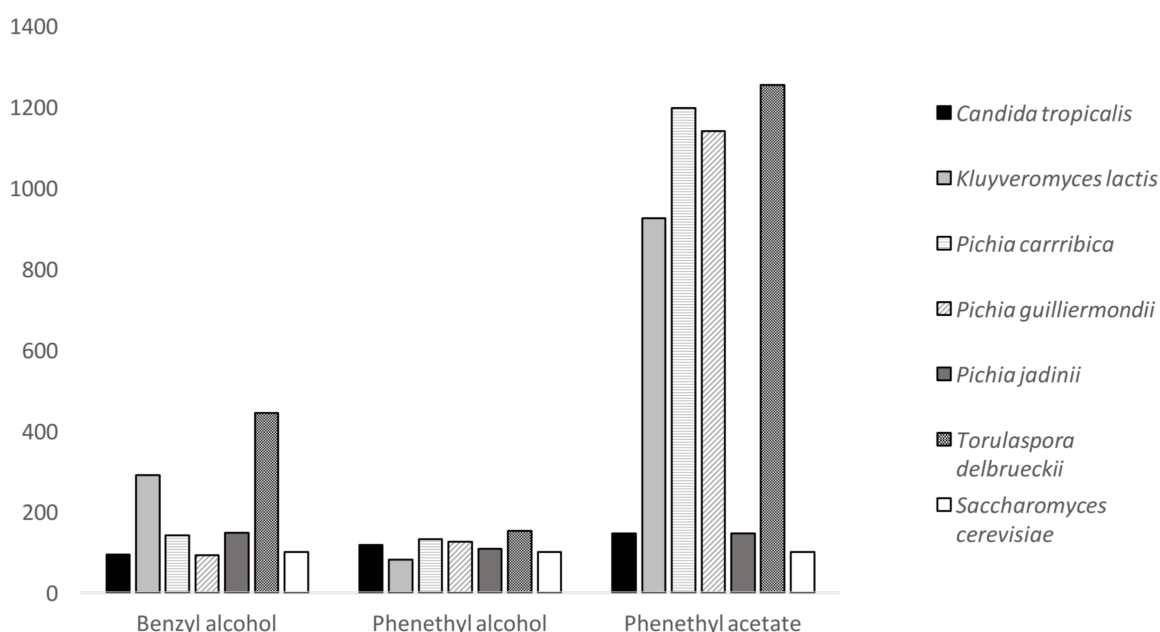

Figure 3b. Normalized peak area of aromatic alcohols and esters (relative to *Saccharomyces cerevisiae* = 100) after 4 weeks of mead fermentation by selected yeast strains. Standard deviations are approximately 15% of mean.

The production of aromatic alcohols benzyl alcohol and phenethyl alcohol, as well as the acetate ester of the latter, is shown in Figure 3b. In sharp contrast to the fatty acid ethyl esters, *Saccharomyces cerevisiae* is the lowest producer of the ester phenethyl acetate. Whereas *C. tropicalis* and *P. jadinii* only produce approximately 50% more of this ester, the yeasts *T. delbrueckii*, *K. lactis, P. guillermondii, P. caribbica* each produce 9-12x the amount. It should be noted that different enzymes are responsible for the production of this ester; in *S. cerevisiae*, alcohol acyltransferases Atf1 and Atf2 control the production of acetate esters such as phenethyl acetate, whereas fatty acid ester production are products of acyl-CoA:ethanol O-acyltransferases Eeb1 and Eht1 (Saerens et al., 2010). It is likely that the high producers of phenethyl acetate either possess a more active variant of the Atf enzymes or regulate their transcription in a different way than *S. cerevisiae*.

The yeasts *K. lactis* and *T. delbrueckii* also produce the aromatic alcohol benzyl alcohol in a significantly higher amount than *S. cerevisiae* (3-4x more). Phenethyl alcohol was produced in a narrower range.

As discussed, floral and fruity notes are associated with high quality honey. The production of these aroma compounds in mead may therefore enhance the intensity of honey aroma in mead. If applied successfully, meads formulations may appear to contain more honey than they actually do, resulting in meads that are cheaper and faster to produce and contain fewer calories.

4 Conclusions

In this study, production of aroma compounds of interest to food fermentations by yeasts from the CCMA collection was a strain-dependent phenomenon. Clustering of yeasts based on correlation between aroma profiles facilitated the selection of yeasts with interesting aroma profiles for alcoholic fermentations. Although several selected yeasts did not grow well on honey must, seven managed to produce sweet, low-alcoholic meads. The aroma profiles that were produced during mead fermentations by *S. cerevisiae* CCMA 1523 and non-conventional yeasts such as *T. delbrueckii* CCMA 1524, *K. lactis* CCMA 1518*, P. guillermondii* CCMA 1521 and *P. caribbica* CCMA 0198 differed especially in the balance of ethyl and acetate esters that were produced. Several of the aroma compounds that the non-*Saccharomyces* yeasts surveyed in this study produced in higher amounts are associated with honey, floral and fruity aromas. They may therefore have the potential to complement the aromas produced by *S. cerevisiae* during alcoholic fermentations such as mead production.

5 References

Amorim, J.C., Schwan, R.F., Duarte, W.F., 2016. Sugar cane spirit (cachaça): Effects of mixed inoculum of yeasts on the sensory and chemical characteristics. Food Res. Int. 85, 76–83. https://doi.org/10.1016/J.FOODRES.2016.04.014

Anupama, D., Bhat, K.., Sapna, V.., 2003. Sensory and physico-chemical properties of commercial samples of honey. Food Res. Int. 36, 183–191. https://doi.org/10.1016/S0963-9969(02)00135-7

Azzolini, M., Tosi, E., Lorenzini, M., Finato, F., Zapparoli, G., 2015. Contribution to the aroma of white wines by controlled Torulaspora delbrueckii cultures in association with Saccharomyces cerevisiae. World J. Microbiol. Biotechnol. 31, 277–293. https://doi.org/10.1007/s11274-014-1774-1

Bang, L.M., Buntting, C., Molan, P., 2003. The Effect of Dilution on the Rate of Hydrogen Peroxide Production in Honey and Its Implications for Wound Healing. J. Altern. Complement. Med. 9, 267–273. https://doi.org/10.1089/10755530360623383

Basso, R.F., Alcarde, A.R., Portugal, C.B., 2016. Could non-Saccharomyces yeasts contribute on innovative brewing fermentations? Food Res. Int. 86, 112–120. https://doi.org/10.1016/J.FOODRES.2016.06.002

Bastos, E.M.A.F., de Souza São Thiago Calaça, P., Simeão, C.M.G., Rodrigues Ribeiro da Cunha, M., 2016. Characterization of the honey from Myracrodruon urundeuva (Anacardiceae-Aroeira) in the Dry Forest of northern of Minas Gerais/Brazil. Adv. Agric. Sci. 4, 64–71.

Bressani, A.P.P., Martinez, S.J., Evangelista, S.R., Dias, D.R., Schwan, R.F., 2018. Characteristics of fermented coffee inoculated with yeast starter cultures using different inoculation methods. LWT 92, 212–219. https://doi.org/10.1016/J.LWT.2018.02.029

Caridi, A., Fuda, S., Postorino, S., Russo, M., Sidari, R., 1999. Selection of Saccharomyces sensu stricto for mead production. Food Technol. Biotechnol. 37, 203–207.

Castro-Vázquez, L., Díaz-Maroto, M.C., González-Viñas, M.A., Pérez-Coello, M.S., 2009. Differentiation of monofloral citrus, rosemary, eucalyptus, lavender, thyme and heather honeys based on volatile composition and sensory descriptive analysis. Food Chem. 112, 1022–1030. https://doi.org/10.1016/J.FOODCHEM.2008.06.036

Daniel, H.-M., Prasad, G.S., 2010. The role of culture collections as an interface between providers and users: the example of yeasts. Res. Microbiol. 161, 488–496. https://doi.org/10.1016/J.RESMIC.2010.01.011

Ducki, S., Miralles-Garcia, J., Zumbé, A., Tornero, A., Storey, D.M., 2008. Evaluation of solid-phase micro-extraction coupled to gas chromatography–mass spectrometry for the headspace analysis of volatile compounds in cocoa products. Talanta 74, 1166–1174. https://doi.org/10.1016/J.TALANTA.2007.08.034

Evangelista, S.R., da Cruz Pedrozo Miguel, M.G., de Souza Cordeiro, C., Silva, C.F.,

Marques Pinheiro, A.C., Schwan, R.F., 2014. Inoculation of starter cultures in a semi-dry coffee (Coffea arabica) fermentation process. Food Microbiol. 44, 87–95. https://doi.org/10.1016/j.fm.2014.05.013

Gamero, A., Quintilla, R., Groenewald, M., Alkema, W., Boekhout, T., Hazelwood, L., 2016. High-throughput screening of a large collection of non-conventional yeasts reveals their potential for aroma formation in food fermentation. Food Microbiol. 60, 147–159. https://doi.org/10.1016/J.FM.2016.07.006

Garg, N., Sethupathy, A., Tuwani, R., NK, R., Dokania, S., Iyer, A., Gupta, A., Agrawal, S., Singh, N., Shukla, S., Kathuria, K., Badhwar, R., Kanji, R., Jain, A., Kaur, A., Nagpal, R., Bagler, G., 2018. FlavorDB: a database of flavor molecules. Nucleic Acids Res. 46, D1210–D1216. https://doi.org/10.1093/nar/gkx957

Gutiérrez, A., Boekhout, T., Gojkovic, Z., Katz, M., 2018. Evaluation of non- *Saccharomyces* yeasts in the fermentation of wine, beer and cider for the development of new beverages. J. Inst. Brew. 124, 389–402. https://doi.org/10.1002/jib.512

Holt, S., Mukherjee, V., Lievens, B., Verstrepen, K.J., Thevelein, J.M., 2018. Bioflavoring by non-conventional yeasts in sequential beer fermentations. Food Microbiol. 72, 55–66. https://doi.org/10.1016/J.FM.2017.11.008

Jung, S.T., Rhim, J.W., Kim, D.H., 1999. Fermentation characteristics of honey wine by saccharomyces bayanus. Korean J. Food Sci. Technol. 31, 1044–1049.

Manyi-Loh, C.E., Ndip, R.N., Clarke, A.M., Manyi-Loh, C.E., Ndip, R.N., Clarke, A.M., 2011. Volatile Compounds in Honey: A Review on Their Involvement in Aroma, Botanical Origin Determination and Potential Biomedical Activities. Int. J. Mol. Sci. 12, 9514–9532. https://doi.org/10.3390/ijms12129514

Mendes-Ferreira, A., Cosme, F., Barbosa, C., Falco, V., Inês, A., Mendes-Faia, A., 2010. Optimization of honey-must preparation and alcoholic fermentation by Saccharomyces cerevisiae for mead production. Int. J. Food Microbiol. 144, 193–198. https://doi.org/10.1016/J.IJFOODMICRO.2010.09.016

Nykänen, L., Nykänen, I., Suomalainen, H., 1977. DISTRIBUTION OF ESTERS PRODUCED DURING SUGAR FERMENTATION BETWEEN THE YEAST CELL AND THE MEDIUM. J. Inst. Brew. 83, 32–34. https://doi.org/10.1002/j.2050-0416.1975.tb03788.x

Oliveira Assis, M., Pereira, A., Santos, C., Rosa, C.A., Eugênia De Oliveira Mamede, M., 2014. Impact of a Non-Saccharomyces Yeast Isolated in the Equatorial Region in the Acceptance of Wine Aroma. Food Nutr. Sci. 5, 759–769. https://doi.org/10.4236/fns.2014.59086

Pereira, A.P., Dias, T., Andrade, J., Ramalhosa, E., Estevinho, L.M., 2009. Mead production: Selection and characterization assays of Saccharomyces cerevisiae strains. Food Chem. Toxicol. 47, 2057–2063. https://doi.org/10.1016/J.FCT.2009.05.028

Pereira, A.P., Mendes-Ferreira, A., Estevinho, L.M., Mendes-Faia, A., 2015. Improvement of mead fermentation by honey-must supplementation. J. Inst. Brew. 121, 405–410.

https://doi.org/10.1002/jib.239

Ravasio, D., Carlin, S., Boekhout, T., Groenewald, M., Vrhovsek, U., Walther, A., Wendland, J., Ravasio, D., Carlin, S., Boekhout, T., Groenewald, M., Vrhovsek, U., Walther, A., Wendland, J., 2018. Adding Flavor to Beverages with Non-Conventional Yeasts. Fermentation 4, 15. https://doi.org/10.3390/fermentation4010015

Ribeiro, L.S., Miguel, M.G. da C.P., Evangelista, S.R., Martins, P.M.M., van Mullem, J., Belizario, M.H., Schwan, R.F., 2017. Behavior of yeast inoculated during semi-dry coffee fermentation and the effect on chemical and sensorial properties of the final beverage. Food Res. Int. 92, 26–32. https://doi.org/10.1016/J.FOODRES.2016.12.011

Saerens, S.M.G., Delvaux, F.R., Verstrepen, K.J., Thevelein, J.M., 2010. Production and biological function of volatile esters in *Saccharomyces cerevisiae*. Microb. Biotechnol. 3, 165–177. https://doi.org/10.1111/j.1751-7915.2009.00106.x

Steensels, J., Meersman, E., Snoek, T., Saels, V., Verstrepen, K.J., 2014. Large-scale selection and breeding to generate industrial yeasts with superior aroma production. Appl. Environ. Microbiol. 80, 6965–75. https://doi.org/10.1128/AEM.02235-14

Steensels, J., Verstrepen, K.J., 2014. Taming Wild Yeast: Potential of Conventional and Nonconventional Yeasts in Industrial Fermentations. Annu. Rev. Microbiol. 68, 61–80. https://doi.org/10.1146/annurev-micro-091213-113025

Sroka, P., Tuszyński, T., 2007. Changes in organic acid contents during mead wort fermentation. Food Chem. 104, 1250–1257. https://doi.org/10.1016/j.foodchem.2007.01.046

Steinkraus, K.H., Morse, R.A., 1973. Chemical Analysis of Honey Wines. J. Apic. Res. 12, 191–195. https://doi.org/10.1080/00218839.1973.11099749

van Breda, V., Jolly, N., van Wyk, J., 2013. Characterisation of commercial and natural Torulaspora delbrueckii wine yeast strains. Int. J. Food Microbiol. 163, 80–88. https://doi.org/10.1016/J.IJFOODMICRO.2013.02.011

World Federation for Culture collections, n.d. Culture Collections Information [WWW Document]. 2014. URL http://www.wfcc.info/ccinfo/index.php/collection/by_id/1083/ (accessed 2.11.19).

Appendix 1

Table 1. Yeast strains (n = 63) from the CCMA culture collection that were included in the screening of volatile organic compounds.

Code	genus	species	origin
CCMA 1514	*Candida*	*frijolensis*	soil
CCMA 0024	*Candida*	*glabrata*	soil
CCMA 1507	*Candida*	*glabrata*	soil
CCMA 0043	*Candida*	*glabrata*	soil
CCMA 0027	*Candida*	*labiduridarum*	soil
CCMA 1505	*Candida*	*neerlandica*	soil
CCMA 1509	*Candida*	*neerlandica*	soil
CCMA 1510	*Candida*	*neerlandica*	soil
CCMA 1515	*Candida*	*neerlandica*	soil
CCMA 0028	*Candida*	*orthopsilosis*	soil
CCMA 0038	*Candida*	*railenensis*	soil
CCMA 0371	*Candida*	*rugosa*	indigenous beverages
CCMA 0040	*Candida*	*sojae*	soil
CCMA 0022	*Candida*	*tetrigidarum*	soil
CCMA 1506	*Candida*	*tetrigidarum*	soil
CCMA 1511	*Candida*	*tetrigidarum*	soil
CCMA 1525	*Candida*	*tetrigidarum*	soil
CCMA 1508	*Candida*	*tropicalis*	soil
CCMA 1512	*Candida*	*tropicalis*	soil
CCMA 1513	*Candida*	*tropicalis*	soil
CCMA 0377	*Candida*	*tropicalis*	indigenous beverages
CCMA 0037	*Cryptococcus*	*flavescens*	soil
CCMA 0035	*Debaryomyces*	*hansenii*	soil
CCMA 0045	*Debaryomyces*	*etchellsii*	silage
CCMA 0153	*Debaryomyces*	*hansenii*	coffee
CCMA 0141	*Debaryomyces*	*polymorphus*	coffee
CCMA 1516	*Hanseniaspora*	*opuntiae*	silage
CCMA 1517	*Issatchenkia*	*orientalis*	silage
CCMA 0048	*Kazachstania*	*exigua*	soil
CCMA 1518	*Kluyveromyces*	*lactis*	silage
CCMA 0348	*Kluyveromyces*	*marxianus*	cocoa
CCMA 0031	*Kodameae*	*ohmeri*	soil
CCMA 0034	*Lindnera*	*saturnus*	soil
CCMA 0192	*Pichia*	*anomala*	coffee
CCMA 0193	*Pichia*	*anomala*	coffee
CCMA 0146	*Pichia*	*anomala*	coffee
CCMA 0149	*Pichia*	*burtonii*	coffee
CCMA 1519	*Pichia*	*capsulata*	silage
CCMA 0198	*Pichia*	*caribbica*	coffee
CCMA 0195	*Pichia*	*fermentans*	cocoa
CCMA 1521	*Pichia*	*guilliermondii*	coffee

Code	genus	species	origin
CCMA 0164	*Pichia*	*guillermondii*	cocoa
CCMA 0144	*Pichia*	*holstii*	silage
CCMA 1520	*Pichia*	*jadinii*	silage
CCMA 0160	*Pichia*	*jadinii*	coffee
CCMA 0237	*Pichia*	*kluyveri*	cocoa
CCMA 0165	*Pichia*	*kluyveri*	cocoa
CCMA 0051	*Pichia*	*kudriavzevii*	silage
CCMA 0379	*Pichia*	*kudriavzevii*	indigenous beverages
CCMA 0382	*Pichia*	*manshurica*	indigenous beverages
CCMA 0048	*Pichia*	*manshurica*	silage
CCMA 0157	*Pichia*	*sydowiorum*	coffee
CCMA 0190	*Saccharomyces*	*cerevisiae*	bio-ethanol
CCMA 1522	*Saccharomyces*	*cerevisiae*	fruits
CCMA 1523	*Saccharomyces*	*cerevisiae*	indigenous
CCMA 0159	*Saccharomyces*	*cerevisiae*	coffee
CCMA 0050	*Schizosaccharomyces*	*pombe*	silage
CCMA 0030	*Schwanniomyces*	*pseudopolymorphus*	soil
CCMA 0033	*Schwanniomyces*	*vanrijiae*	soil
CCMA 0372	*Torulaspora*	*delbrueckii*	indigenous beverages
CCMA 1524	*Torulaspora*	*delbrueckii*	coffee
CCMA 0029	*Trichosporon*	*loubieri*	soil
CCMA 0175	*Zygosaccharomyces*	*bailii*	silage

Appendix 2

Table 1. Yeast strains (n=19) that were selected for inclusion in the mead follow-up experiment, based on performance in the screening of volatile organic compounds.

Strain	Origin	Compound of interest
Candida tropicalis (CCMA 0377)	indigenous beverage	Ethyl hexanoate
Debaryomyces hansenii (CCMA 0035)	soil	Phenyl ethanol
Debaryomyces polymorphus (CCMA 0141)	coffee	Ethyl hexanoate
Hanseniaspora opuntiae (CCMA 1516)	silage	Phenyl ethyl acetate, phenyl ethanol
Issatchenkia orientalis (CCMA 1517)	silage	Phenyl ethanol
Kazachstania exigua (CCMA 0048)	soil	Phenyl ethanol
Kluyveromyces lactis (CCMA 1518)	silage	Phenyl ethyl propanoate, phenyl ethanol
Kluyveromyces marxianus (CCMA 0348)	cocoa	Benzyl alcohol
Pichia caribbica (CCMA 0198)	coffee	Phenyl ethyl propanoate, phenyl ethanol
Pichia guilliermondii (CCMA 1521)	coffee	Benzyl benzoate, phenyl ethanol
Pichia jadinii (CCMA 0160)	coffee	Ethyl hexanoate, phenyl ethanol
Pichia kluyveri (CCMA 0165)	cocoa	Phenyl ethyl acetate
Pichia kluyveri (CCMA 0237)	cocoa	Phenyl ethyl acetate
Pichia kudriavzevii (CCMA 0379)	indigenous beverage	Phenyl ethanol
Pichia manshurica (CCMA 0048)	silage	phenyl ethanol, ethyl hexanoate
Saccharomyces cerevisiae (CCMA 1523)	indigenous beverage	Phenyl ethanol
Schwanniomyces pseudopolymorphus (CCMA 0030)	soil	Benzyl benzoate
Torulaspora delbrueckii (CCMA 1524)	coffee	Phenyl ethyl propanoate
Zygosaccharomyces bailii (CCMA 0175)	silage	Phenyl ethanol, benzyl alcohol

Appendix 3

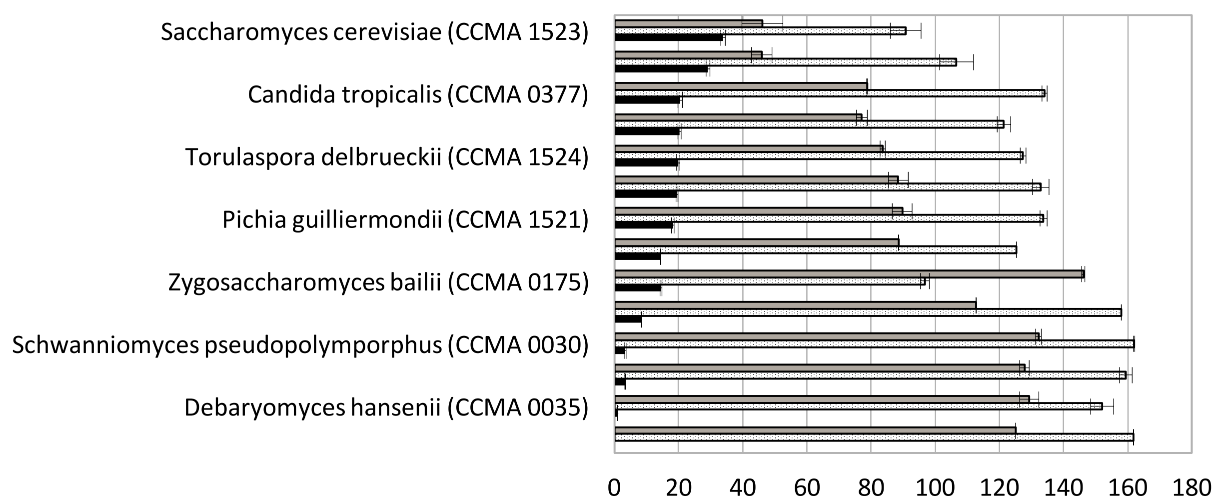

Figure 1. Residual concentrations (in g l^{-1}) of glucose (gray bars), fructose (dotted bars) and production of ethanol (dashed bars) in mead after two weeks of fermentation by selected strains

The yeasts *Kazachstania exigua* (CCMA 0048), *Hansienaspora opuntiae* (CCMA 1516), *Debaryomyces polymorphus* (CCMA 0141), *Pichia kudriavzevii* (CCMA 0379) and *Kluyveromyces marxianus* (CCMA 0348) were included in the selection but did not grow on honey must.

Appendix 4

Table 1. Peak areas (logarithmic values) of compounds detected by GC-MS in mead must and meads after 4 weeks of fermentation by selected yeasts*

	Must	*C. tropicalis*	*P. jadinii*	*S. cerevisiae*	*K. lactis*	*T. delbrueckii*	*P. guilliermondii*	*P. carribica*
1-Butanol, 3-methyl-		6,04	6,04	5,97	6,05	6,05	5,69	5,97
Octanoic acid, ethyl ester		4,27	5,03	5,18	4,09	4,13	4,24	4,47
Acetic acid	4,76	6,47	5,85	6,00	5,97	6,00	6,08	5,77
Benzaldehyde	6,00	4,69	4,86	4,86	4,70	4,58	4,96	5,04
Nonanoic acid, ethyl ester		3,73	3,84	4,59	3,63	3,53	3,97	4,09
Linalool	3,90	4,21	4,05	3,95	4,19	4,18	4,01	4,25
Propanoic acid, 2-methyl-	4,27	4,43	4,40	4,55	4,49	4,77	4,66	4,52
2,3-Butanediol		5,07	4,81	5,36	4,52	4,85	4,65	4,67
Hotrienol	3,78	3,54	3,77	3,74	4,31	4,52	3,79	4,01
Decanoic acid, ethyl ester		4,59	4,69	5,68	4,34	4,31	4,48	4,63
Butanoic acid, 3-methyl-	5,18	4,98	4,62	4,67	4,69	4,71	4,58	4,58
Ethyl 9-decenoate		3,57	3,55	4,45	3,34	3,61	4,11	4,12
Epoxylinalol	4,35	3,88	3,92	3,80	3,99	4,10	3,95	4,10
2-Undecenal	4,08			4,01				
1-Decanol		4,09	4,06	3,85	3,90	3,98	3,96	3,92
Acetic acid, 2-phenylethyl ester		5,59	5,59	5,42	6,39	6,52	6,48	6,50
Dodecanoic acid, ethyl ester		4,75	4,33	5,51	4,64	4,46	4,71	4,73
Benzyl alcohol	4,62	5,38	5,57	5,40	5,87	6,05	5,37	5,55
Benzyl nitrile	4,23							
Phenylethyl Alcohol	5,46	7,05	7,01	6,98	6,89	7,16	7,08	7,10
Hexanoic acid, 2-ethyl-		3,78	4,23		4,05	4,05		
Benzenemethanol, 4-methyl-				3,69				4,63
1-Dodecanol	4,06	4,45	4,62	5,11	4,61	4,69	4,74	4,61
p-Anisaldehyde	6,72	5,76	6,05	5,54	5,61	5,58	5,85	5,84
Isopropyl myristate		4,18	4,09		4,11	4,26	3,93	3,65
Ethyl tetradecanoate		4,44	4,43	5,20	4,95	4,85	5,21	5,16
Octanoic acid	4,64	5,10	5,28	4,77	4,49	4,54	4,58	4,52
Benzyl alcohol, 3,5-dimethyl-		3,93	4,02	3,03	4,41	4,24	3,25	3,58
Ethyl p-anisoate		4,88	4,98	4,75	4,90	4,85	4,85	4,84
Pentadecanoic acid, ethyl ester		3,94	4,07	4,22	4,09	3,98	4,05	3,81
.gamma.-n-Decalactone			3,84		4,02		3,90	4,04
Nonanoic acid	4,54	4,52	4,69	4,02	4,56	4,50	4,24	4,31
Ethyl 4-methoxyphenylacetate	3,84	5,63	5,70	5,63	5,64	5,77	5,56	5,60
Hexadecanoic acid, ethyl ester	4,37	5,58	5,26	5,74	5,38	5,26	5,49	5,53
n-Decanoic acid	4,24	4,53	4,77	4,43	4,41	4,36	4,16	4,22
4-Methoxyphenethyl alcohol	3,89	5,12	5,02	5,07	4,91	5,21	5,00	5,13
1-Hexadecanol	3,80	4,03	4,49	3,73	4,50	4,38	4,02	3,74
Octadecanoic acid, ethyl ester	3,87	4,81	4,72	4,70	4,75	4,54	4,79	4,75
Benzoic acid	4,17	4,50	4,56	4,60	4,39	4,43	4,03	4,39
Ethyl Oleate	3,58	4,22	4,51	4,59	4,60	4,40	4,71	4,47
Dodecanoic acid	3,43	4,07	4,14	3,86	4,15	3,87	3,47	3,60
Linoleic acid ethyl ester	3,16	3,71	3,92	4,24	4,00	3,59	3,79	3,88
Diisobutyl phthalate	5,32	5,42	5,77	4,97	5,77	5,51	5,57	5,37
Dibutyl phthalate	3,67	4,08	4,42	3,93	4,35	4,10	4,19	4,17
Benzyl Benzoate	3,70	3,74	3,71	4,10	4,03	3,74	3,86	3,90
Phthalic acid, di(2-methylbutyl) ester	4,06	4,41	4,40	3,79	4,54	4,19	4,27	4,21
Tetradecanoic acid	3,84	4,31	4,45	4,15	4,37	4,06	4,03	4,16
Pentadecanoic acid			3,97					
n-Hexadecanoic acid	4,00	4,68	4,62	4,41	4,57	3,98	4,26	4,17

* C. tropicalis = *Candida tropicalis* CCMA 0377*,* P. jadinii = *Pichia jadinii* CCMA 0160*,* S. cerevisiae = *Saccharomyces cerevisiae* EU60.1, K. lactis = *Kluyveromyces lactis CCMA 1518,* T. delbrueckii = *Torulaspora delbrueckii CCMA 1524,* P. guilliermondii = *Pichia guilliermondii* CCMA 1521*,* P. carribica = *Pichia caribbica* CCMA 019

ARTICLE 2: PRODUCTION OF LOW-ALCOHOLIC MEAD WITH NON-CONVENTIONAL YEASTS IMPROVES AROMA PROFILE AND SENSORY QUALITY

This article has been formatted according to the guidelines of the journal *Food microbiology*.

Abstract

In recent years, ample research has focused on the application of non-conventional yeasts in the production of alcoholic beverages. Demonstrated benefits of selected non-conventional yeast strains include simultaneous high production of fruity and floral aroma compounds and low production of ethanol. In this contribution, strains of *Saccharomyces cerevisiae* as well as the non-conventional yeasts *Pichia jadinii*, *Torulaspora delbrueckii* and *Kluyveromyces lactis* were applied in the production of mead, which is a honey-based alcoholic beverage. Both single culture fermentations and mixed culture fermentations of *S. cerevisiae* with the other strains were carried out. To evaluate the quality of meads produced in this way, consumption of sugars and production of ethanol, glycerol and organic acids were analyzed with high-performance liquid chromatography. The volatile compound composition was determined with gas chromatography – mass spectrometry to identify aroma-active compounds. A tasting panel evaluated the intensity of selected taste and flavor attributes and hedonic appreciation. Meads with various amounts of ethanol (4.7–11.0 % v/v) and residual sugar contents (70.81-160.25 g l^{-1}) were produced. In both single strain and mixed strain fermentations with *S. cerevisiae*, meads produced with either *Torulaspora delbrueckii* or *Kluyveromyces lactis* had a roughly three-fold higher content of honey-aroma compound phenethyl acetate and a higher acceptance rate than meads produced with only *S. cerevisiae*. Moreover, the sensory panel expressed a preference for low-alcoholic meads with high residual sweetness. These results demonstrate the potential of non-conventional yeasts to improve the aroma profile and sensory quality of low-alcoholic meads.

Keywords: alcoholic fermentations, mead, honey, non-conventional yeasts, *Torulaspora delbrueckii*, *Kluyveromyces lactis*

1 Introduction

Mead is a traditional, honey-based beverage. Historically, it was among the most commonly consumed alcoholic drinks among the Egyptian, Ancient Greek and Roman civilizations as well as in Medieval Europe (Vidrih and Hribar, 2016). Up until today, its consumption is widespread in several Eastern European countries as well as Russia. Despite the economic importance of honey to Brazil (ABEMEL, 2018), there is currently little domestic mead production.

The beverage is produced by fermentation of a diluted honey solution, with optional addition of salts that support the growth of yeasts. The ratio of honey to water in the initial must can vary from 2:1 to 1:3 or less, each producing distinct types of mead (Ramalhosa et al., 2011). The final beverage can have an alcohol content between 4-18% v/v depending on the initial concentration of honey and extent of fermentation. Meads can be dry or sweet, with residual sugar concentrations ranging from 2.5-28% w/w (Steinkraus and Morse, 1973).

The main role of yeasts during the production of mead is to break down the fermentable sugars from honey into ethanol. In the process, a variety of compounds are produced including higher alcohols and esters. In an earlier study, 16 yeast-derived metabolites that contribute to the sensory quality of mead (Mendes-Ferreira (2010). Other studies established that aroma production by *Saccharomyces cerevisiae* starter cultures in mead was influenced by nitrogen supplementation (Pereira et al., 2015) and inoculum size (Pereira et al., 2013).

In recent years, a growing interest in non-conventional yeast species (defined as yeasts other than *S. cerevisiae*), has spurred research on their application in alcoholic beverages. Among the reported benefits of using such yeasts is that some of them produce floral and fruity aroma compounds that are relevant to alcoholic fermentations in significantly higher concentrations than commercial strains of *S. cerevisiae* (Gamero et al., 2016; Gutiérrez et al., 2018). Selecting yeasts with a high production of certain desirable aroma compounds, such as ethyl and acetate esters, can therefore be used to modulate the aroma profile of beer, wine and other beverages (Basso et al., 2016; Dashko et al., 2015; Jolly et al., 2017). Another stated objective is to lower the alcohol content of these beverages, without compromising the intensity of aromas that are normally produced in these fermentation processes (Quirós et al., 2014; Varela et al., 2016).

Potential downsides of using non-conventional yeasts to produce alcoholic beverages is that excessively high concentrations of certain aroma-active compounds can be perceive as

overwhelming or lead to imbalances in the aroma palette (Lilly et al., 2006); or that incomplete fermentations lead to high residual sweetness. Mead may be a suitable application for non-conventional yeasts, as floral and fruity aromas that are often produced by non-conventional yeasts are considered desirable in this product and are associated with high-quality honey (Anupama et al., 2003). Furthermore, residual sugars are commonly present in commercial meads, which are often quite sweet.

In this study, a strain of *S. cerevisiae* as well as three non-conventional yeast strains were used as starter cultures, as single strains or in co-culture with *S. cerevisiae*. Fermentation performance was evaluated by following their consumption of sugars and production of ethanol, glycerol and organic acids. Production of aroma compounds was assessed by gas chromatography – mass spectrometry. Finally, a sensory evaluation was performed to determine the intensity of selected taste and odor attributes as well as overall appreciation of the meads.

All yeasts that were used in this study were selected on the basis of a previous aroma screening of yeasts in the Culture Collection of Agricultural Microbiology (CCMA), a Brazilian culture collection that is specialized fermentation processes. During a preliminary screening, the selected strains produced high amounts of food-relevant aroma compounds and were proven capable of producing low-alcoholic meads. The first objective of this work is to investigate how non-conventional yeast starter cultures (applied as single cultures or in co-culture with *S. cerevisiae*) modulate the aroma profile of mead. A second objective is to evaluate their suitability to produce low-alcoholic meads with pleasant sensorial characteristics.

2 Materials and methods

2.1 Yeasts

The yeasts that were used in this study were *Saccharomyces cerevisiae* CCMA 1523, *Torulaspora delbrueckii* CCMA 1524, *Kluyveromyces lactis* CCMA 1518 and *Pichia jadinii* CCMA 0160. They were used in single culture fermentations and in mixed culture fermentations of *S. cerevisiae* and each of the other strains individually. All strains come from the Culture Collection of Agricultural Microbiology (CCMA) of the Federal University of Lavras (UFLA) that is based in Minas Gerais, Brazil. The culture collection is registered under number 1083 at the World Federation of Culture Collections.

2.2 Storage and re-activation

Yeast stocks are stored at -80 °C in 20% (w/v) glycerol. For reactivation, a loop of the stock material is streaked on an YEPG agar plate (1% (w/v) yeast extract, 2% soy peptone, 2% glucose, 2% agar) and incubated at 28 °C for at least 16h, depending on the growth of the yeast.

2.3 Honey must fermentations

Honey produced by *Apis mellifera* from *Myracrodruon urundeuva*, locally known as aroeira honey (Bastos et al., 2016), was obtained from a beekeeper in Taiobeiras, Minas Gerais, Brazil. Meads were prepared in a similar way as described by Sroka and Tuszynski (2017), with modifications. The honey was diluted with mineral water (Ingai, Brazil) until the solution reached a Brix reading of 24 degrees. The must was supplemented with 0.45 g/l of diammonium phosphate (DAP) to prevent nitrogen limitation. No salts or vitamins were added, as benefits ascribed to these supplements lack empirical support (Pereira et al., 2015). The must was pasteurized in a water bath at 60 °C for 25 min. One and a half liter bottles were equipped with an airlock and filled with 1 l of honey must. Pre-cultures were made by inoculating re-activated yeasts of selected strains from a single colony in the honey must and incubating for 24 hours at 28 °C. The honey must was inoculated from pre-cultures to achieve a cellular density of 10^5 cfu /ml, based on cell counts in a Neubauer chamber. For mixed inoculations, half of this amount was added for each of the strains. The bottles were incubated at 25 °C. Fermentations were monitored by CO_2 release (weight loss of the bottles) and were completed after 36 days, until the weight of all bottles differed by less than 2 g after measuring at two-day intervals. Samples (15 ml) were taken after 24h, 48h, then at 48h intervals (first 10 days), then at 96h intervals until the end of fermentation for analysis of organic acids, sugars and ethanol content (all timepoints) and volatile composition (final timepoint). Samples were frozen at -18 °C until further analysis. Fermentations were carried out in duplicate but at each intermediate time point, samples were taken from only one bottle (alternating) to limit the impact of sampling on the fermentation process.

2.4 Solid phase micro-extractions

Volatile compounds were extracted as described by Ribeiro et al. (2017) with minor modifications, using a manual headspace-solid phase micro-extraction procedure (HS–SPME) with a divinylbenzene/carboxen/polydimethylsiloxane 50/30 µm SPME fiber (Supelco Co., Bellefonte, PA, USA). Two ml of liquid sample was mixed with 0.5 g of sodium chloride, to

improve extraction efficiency (Ducki et al., 2008), and placed in a 15 ml hermetically sealed vial. After equilibration at 60 °C for 15 min, the volatile compounds were extracted at 60 °C 30 min. Desorption time on the column was 3 min.

2.5 Gas chromatography – mass spectrometry

Samples were analyzed by gas chromatography – mass spectrometry (GC-MS) to determine their volatile compound composition. Operating conditions were as described by Ribeiro et al. (2017). Compound identification was based on comparison of mass spectra to the NIST 11 library and comparison of retention index based on an alkane series to data reported in the literature, as described by Bressani et al. (2018).

2.6 High performance liquid chromatography

Samples were analyzed by high performance liquid chromatography to determine the concentration of residual sugars, ethanol and organic acids. They were prepared by centrifuging twice at 9000 rpm, 4 °C for 10 min and filtering the second supernatant with a 0.22 μm nitrocellulose membrane. HPLC operating conditions, compound identification and quantification were as described by Evangelista et al. (2014) with minor modifications. A Shimadzu liquid chromatography system (Shimadzu Corp., Japan) equipped with a dual detection system consisting of a UV–Vis detector (SPD 10Ai) and a refractive index detector (RID-10Ai) is used. A Shimadzu ion exclusion column, Shim-pack SCR-101 H (7.9 mm x 30 cm) was operated at 30 °C. A perchloric acid solution with pH 2.11 was used as the eluent at a flow rate of 0.6 ml/min. Compounds were detected via RID (sugars, ethanol) or UV-Vis (organic acids).

2.7 Data analysis

GC-MS data were analysed using OpenChrom 1.3.0 and AMDIS. Identifications were based on mass spectra from NIST 11 and Kovat's retention index. Statistical analyses (ANOVA and Tukey's HSD post-hoc test) were performed using Python with Statsmodels. A principal component analysis and heat map were produced with Clustvis (https://biit.cs.ut.ee/clustvis/) (Metsalu and Vilo, 2015).

2.8 Sensory analysis

A panel was composed of twenty-one untrained panellists aged 21-58, both male and female, who indicated that they enjoy drinking alcoholic beverages. Meads were served at

room temperature using 15 ml servings in plastic cups. Samples were assigned random numeric codes and served in random order. Samples were evaluated in two rounds, as described by Dashko et al. (2015). Panellists were asked to taste each sample first and rank their overall impression on a structured 9-point hedonic scale (Peryam and Pilgrim, 1957). Afterwards, each sample was tasted again to score taste attributes (sweet, sour) and flavour attributes (alcoholic, fruity, honey, floral, herbal, woody and intensity) on an intensity scale from 1-9. Attributes were based on those proposed by Castro-Vaszquez et al. (2008) to describe the sensory characteristics of honey, but were adjusted to mead samples. Panellists rinsed their mouth with water in between samples.

3 Results and discussion

3.1 Consumption of sugars and production of ethanol, glycerol and organic acids

In this study, mead was produced using *S. cerevisiae* and the non-conventional yeasts *T. delbrueckii*, *K. lactis* and *P. jadinii* as single strain starter cultures. Each of the non-conventional yeast strains was also applied in co-culture with *S. cerevisiae*. The fermentation process was monitored by release of CO_2 (Appendix 1). This revealed that the fermentations had lag phases between 24-96 hours and took 36 days to complete. CO_2 release kinetics for mixed starter cultures were more similar to single strain cultures of *S. serevisiae* than the non-conventional strains that they contained.

Upon completion of fermentation, consumption of sugars and production of glycerol and ethanol were determined (Table 1). The must contained the fermentable sugars glucose, fructose and maltose in a total concentration of 243.41 g l^{-1}. Honey of *Myracrodruon urundeuva* flowers is known to contain negligible amounts of sucrose due to the presence of invertase in the honey (Bastos et al., 2016) and in the must prepared for this study it was not detected. All yeasts showed a slight preference for consumption of glucose over fructose, as is commonly observed for *Saccharomyces*, *Pichia* and *Torulaspora* wine yeasts (Mestre Furlani et al., 2017; Tronchoni et al., 2009). Maltose remained mostly unfermented.

The mead beverages contained between 70.81 g l^{-1} and 160.25 g l^{-1} of residual sugars and between 37.48 g $l^{-1}$and 87.15 g l^{-1} of ethanol (corresponding to 4.7-11.0% v/v). The highest concentration of ethanol was found in meads produced with *Pichia jadinii*, although it was not significantly different from meads obtained with *Saccharomyces cerevisiae* and mixed starter cultures. Meads obtained with single strain starter cultures of *T. delbrueckii* and *K. lactis* contained the lowest amount of ethanol and a significantly higher amount of residual

glucose than all other samples. These samples also released the lowest amounts of carbon dioxide.

Table 1. Concentrations (g l^{-1}) of sugars and alcohols in honey must and in mead after 36 days of fermentation. SC = *Saccharomyces cerevisiae*, PJ = *Pichia jadinii*, TD = *Torulaspora delbrueckii*, KL = *Kluyveromyces lactis*.

	must	SC	PJ	TD	KL	SC + PJ	SC + TD	SC + KL
Glucose	92.33±2.56	15.04±4.70 b	5.22±0.39 b	48.93±8.66 a	38.89±8.00 a	6.72±1.21 b	15.68±4.04 b	15.71±4.28 b
Fructose	115.16±3.24	42.66±8.42 bcd	33.79±3.92 cd	81.02±6.93 ab	70.33±9.84 abc	33.99±0.23 cd	42.28±8.74 bcd	42.99±8.05 bcd
Maltose	35.92±0.88	30.37±4.10	32.17±5.56	30.30±1.89	29.48±2.23	30.10±1.56	30.52±3.37	29.68±1.11
Ethanol	nd	71.11±7.16 ab	87.15±17.03 a	37.48±8.21 b	46.83±1.38 b	68.68±13.54 ab	73.01±6.43 ab	67.63±2.72 ab
Glycerol	nd	5.49±0.97 a	4.98±0.57 ab	3.65±0.41 ab	2.75±0.78 b	4.66±0.42 ab	5.72±0.60 a	5.05±0.48 ab

nd = not detected; different letters in same row mean samples are significantly different according to One-way ANOVA with Tukey's HSD post-hoc test ($P<0.05$).

The high ethanol production of *Pichia jadinii* is a remarkable result. While strains of *S. cerevisiae* usually produce higher amounts of ethanol than non-conventional yeasts, previous studies identified strains non-conventional yeasts of species including *Pichia kudriavzevii* and *Pichia anomala* as high ethanol producers, some with potential for bio-ethanol production (Ruyters et al., 2015; Zha et al., 2013). Furthermore, the strain of *Saccharomyces cerevisiae* that was utilized in this study (CCMA 1523) is a wild strain that was originally isolated from a low-alcoholic cassava fermentation.

The main organic acids that were produced during the fermentation were citric acid (6.00-12.33 g l^{-1}) and acetic acid (0.24-0.38 g l^{-1}). Succinic acid and malic acid were present in the must and persisted during the fermentation, although the concentration of the latter decreased over time (Appendix 2).

Mead is a beverage that can be produced in a variety of styles. Previous studies on the composition of commercial meads found that most meads contain approximately 13% v/v of ethanol (in a range from 6.3 – 20.8% v/v). Residual sugar concentrations found in commercial meads varied widely, in a range of 25 – 278 g l^{-1} (Šmogrovičová et al., 2012; Steinkraus and Morse, 1973). The mead beverages that were produced for this study can

therefore be characterized as low alcoholic, while the amounts of residual sugar are within the typical range for commercial meads.

3.2 Production of aroma compounds

Volatile organic compounds that were present in the meads obtained in this study were analyzed using gas chromatography – mass spectrometry (Table 2). A total of 40 compounds was detected, of which 19 also occurred in the must. The most important classes were esters (14), acids (8) and alcohols (6). Flavor descriptors of each compound, taken from FlavorDB (Garg et al., 2018), are featured in Appendix 3.

In comparison with *S. cerevisiae*, both *T. delbrueckii* and *K. lactis* produced over three times more phenethyl acetate than *S. cerevisiae*, but produced lower amounts of fatty acid ethyl esters such as ethyl octanoate and ethyl decanoate. This could result in different aroma profiles as phenethyl acetate contributes to floral aromas (tobacco, rose) whereas ethyl octanoate and ethyl decanoate contribute to fruity aromas (apricot and apple).

The production of fatty acid ethyl esters and acetate esters are controlled by different genes (Saerens et al., 2010) and regulation of these genes may be strain-dependent. Previous studies confirmed the ability of specific *T. delbrueckii* strains to produce phenethyl acetate in higher quantities than *S. cerevisiae* wine strains (Loira et al., 2014) while others found opposite results (Chen and Liu, 2016; Viana et al., 2008b).

The yeast *T. delbrueckii* was also characterized by absence of 2,3-butanediol (buttery notes) and low production of 3-methyl-1-butanol, which can cause off-flavors at sufficiently high concentrations (Bartowsky and Pretorius, 2009).

Like *S. cerevisiae, P. jadinii* produced relatively high amounts of fatty acid ethyl esters and low amounts of acetate esters. In comparison with *S. cerevisiae*, it produced lower amounts of several important aroma compounds, including phenethyl alcohol (rose aroma) and γ-decalactone (peach aroma).

The volatile composition of meads produced with mixed inoculations contain characteristics of the volatile fingerprint of both *S. cerevisiae* and the non-conventional yeasts. For example, the mixed fermentations of *S. cerevisiae* with *T. delbrueckii* and *K. lactis* both contained high amounts of ethyl octanoate (characteristic of *S. cerevisiae*) and phenethyl acetate (characteristic of *T. delbrueckii* and *K. lactis*). This indicates that the aroma profile of meads can, in fact, be enriched by the use of mixed fermentations in comparison with single strain fermentations.

A number of aroma compounds was produced in higher amounts by mixed starter

cultures than by single strain starter cultures. For example, the mixed starter culture of *S. cerevisiae* and *K. lactis* produced higher amounts of several ethyl esters and lactones than *S. cerevisiae* or *K. lactis* produced individually. Production of ethyl octanoate by this mixed starter culture was four times higher than that of single strain *K. lactis* and 58% higher than single strain *S. cerevisiae*.

A principal component analysis performed on the volatile composition of meads (Figure 1) groups meads produced with *S. cerevisiae* in a single cluster with all meads produced with mixed starter cultures, separated from single strain starter cultures of *P. jadinii*, *T. delbrueckii* and *K. lactis* along PC1 (explaining 60.8% of variance). Single strain starter cultures of *T. delbrueckii* and *K. lactis* are separated from *P. jadinii* along PC2 (15.9% of variance) and to a lesser extent this also separated mixed starter cultures containing these strains. This shows that despite the influence of non-conventional yeast strains on the volatile composition of meads produced with mixed starter cultures, their volatile profiles were more similar to that of *S. cerevisiae*.

Adding complexity to the aroma profile is one of the main reasons to use mixed starter cultures. This can occur if both strains persist during at least part of the fermentation and carry out their specific metabolic activities (Ciani and Comitini, 2015). In that case both species are able to add metabolites to the overall aroma profile, as is observed for phenethyl acetate and ethyl octanoate in this study, for mixed fermentations with *S. cerevisiae* and *T. delbrueckii* or *K. lactis*.
In some cases, mixed cultures produce higher or lower amounts of specific metabolites than may be expected on the basis of single strain fermentations, for example due to synergistic effects caused by extracellular enzymes (Maturano et al., 2012) or shifts in the extracellular environment caused by the co-inoculated strain (Zara et al., 2014).

3.3 Sensory analysis

A sensory analysis was performed to evaluate the intensity of several taste and flavor attributes in the meads, as well as hedonic overall appreciation by the panelists. Results are shown in a way that enables separate comparisons between single strain starter cultures (Figure 3a) and mixed starter cultures (Figure 3b). Differences were more pronounced and more often statistically significant between single strain starter cultures than between mixed starter cultures. This matches the observation from the principal component analysis that meads produced with mixed starter cultures had more similar volatile compound compositions than those produced with single strains.

Mead produced with a single strain starter culture of *T. delbrueckii* obtained the highest score for overall appreciation and balance, followed by mead produced with *K. lactis*. Panelists identified these meads as sweeter, less sour and less alcoholic than other mead samples, in agreement with the actual composition of sugars, ethanol and acids that are reported in this study.

The higher intensity of honey aroma in samples produced with *T. delbrueckii* and *K. lactis* can alternatively be explained by the higher amount of residual sugars from honey that is present in these samples or their higher production of honey-aroma compound phenethyl acetate. However, a higher intensity honey aroma was not perceived in meads produced with mixed cultures of *S. cerevisiae* with *T. delbrueckii* or *K. lactis*, while these contained similar amounts of this aroma compound.

The high ANOVA significance level of attributes sweetness and alcoholic ($P < 0.001$) may indicate that panelists were more familiar with these descriptors. Untrained panels often find it difficult to distinguish specific odor attributes in complex beverages (Hopfer and Heymann, 2014), perhaps explaining why perceived differences for odor attributes such as fruity, floral and herbal were not statistically significant in this study.

Overall appreciation of the meads varied greatly among the panelists. Each of the samples received both very low scores (1 or 2) and very high scores (8 or 9), prompting a closer investigation of the distribution of these scores. To this end, individual panelist's preferences were recorded in a heatmap (Figure 3) and clustered by Euclidian distance. In this heatmap, red fields indicate high hedonic appreciation whereas blue fields indicate low appreciation.

The clustering revealed the presence of four groups of tasters: (i) those who liked most or all of the meads (rows 1-5); (ii) those who disliked most or all meads (rows 14-17); (iii) those who liked only the sweetest meads, produced without *S. cerevisiae* (rows 18-21) and finally, (iv) those who liked only some of the meads, including at least one of the meads produced with *S. cerevisiae* (rows 5-13).

This perspective reveals that mead produced with *T. delbrueckii* appealed to the largest group of tasters; 16 out of 21 panelists (76%) awarded it 6 (out of 9) or more points for overall appreciation. By the same metric, mead produced with only *S. cerevisiae* appealed to the smallest group of users (24%). The mead produced with a mixed starter culture of *S. cerevisiae* and *T. delbrueckii* appealed to 62% of the panelists, outperforming those produced with other mixed starter cultures.

Previous studies on the influence of sweetness and ethanol content on mead acceptability also found that the sweetest meads that were included in the study had the highest acceptability (Gomes et al., 2015; Vidrih and Hribar, 2007). Sweetness is an important characteristic of mead and depends on both the initial ratio of honey to water that is used and the extent of fermentation. Preferences may differ by region; in one study, commercial meads from South-Africa contained 67-77 g/l of residual sugar on average whereas commercial Slovak meads contained 137-200 g/l (Šmogrovičová et al., 2012). The Brazilian panelists who evaluated this study's meads may share the preference for sweeter meads, or dryer mead may simply be an acquired taste to which they had no previous exposure.

Table 2. Volatile organic compounds detected in mead samples by HS-SPME/GC-MS (logarithmic peak areas, mean±sd). SC = *Saccharomyces cerevisiae*, PJ = *Pichia jadinii*, TD = *Torulaspora delbrueckii*, KL = *Kluyveromyces lactis*.

Compound	Must	*SC*	*PJ*	*TD*	*KL*	*SC + PJ*	*SC + TD*	*SC + KL*	ANO-VA
Alcohols									
1-butanol, 3-methyl		6.14±0.25 ab	5.96±0.15 ab	5.59±0.07 b	5.82±0.05 ab	6.09±0.18 ab	5.81±0.18 ab	6.21±0.00 a	*
2,3-butanediol		4.75±0.31 a	4.38±0.00 a	nd	4.22±0.18 a	5.05±0.20 a	4.89±0.10 a	4.80±0.06 a	***
benzyl alcohol	X	6.13±0.20	4.97±0.10	5.49±0.05	5.80±0.05	5.87±0.59	5.41±0.17	5.71±0.59	NS
phenethyl alcohol	X	7.29±0.03	6.99±0.09	7.08±0.22	7.08±0.16	7.48±0.11	7.45±0.14	7.46±0.08	*
p-anisylalcohol	X	5.17±0.04	4.60±0.26	5.27±0.37	5.38±0.56	5.61±0.21	5.47±0.39	5.24±0.03	NS
2-(4-methoxyphenyl)-ethanol		5.09±0.04	4.58±0.00	4.84±0.45	4.68±0.51	5.48±0.12	5.35±0.39	5.15±0.25	NS
Acids									
butanoic acid, 3-methyl	X	5.22±0.00	4.81±0.10	4.83±0.07	4.92±0.03	5.18±0.05	4.92±0.16	5.35±0.46	NS
benzeneacetic acid, 4-methoxy		5.78±0.13 a	5.36±0.00 ab	4.88±0.34 b	5.59±2.30 ab	5.94±0.05 a	5.45±0.26 ab	5.90±0.15 a	**
octanoic acid	X	6.27±0.09 a	6.04±0.13 a	5.97±0.17 ab	5.34±0.34 b	6.57±0.11 a	6.20±0.06 a	6.36±0.14 a	**
nonanoic acid	X	5.22±0.19	4.76±0.27	4.88±0.23	4.82±0.37	5.29±0.16	4.95±0.18	5.27±0.05	NS
n-decanoic acid	X	6.26±0.15 ab	6.10±0.17 ab	5.74±0.30 bc	5.10±0.19 c	6.67±0.28 a	6.20±0.01 ab	6.31±0.06 ab	**
benzoic acid	X	5.43±0.00	4.45±0.03	5.25±0.97	4.63±0.13	5.38±0.04	5.12±0.22	4.84±0.25	NS
dodecanoic acid	X	5.48±0.07 ab	5.13±0.16 ab	5.10±0.31 ab	4.92±0.20 b	5.77±0.25 a	5.37±0.03 ab	5.61±0.08 ab	*
tetradecanoic acid	X	4.70±0.26	4.33±0.09	4.18±0.44	4.23±0.32	4.79±0.37	4.63±0.08	4.72±0.07	NS
Esters									
octanoic acid, ethyl ester		5.61±0.08 ab	5.70±0.08 ab	5.27±0.33 ab	5.19±0.20 b	5.95±0.03 a	5.68±0.00 ab	5.81±0.25 ab	*
decanoic acid, ethyl ester		6.12±0.00 a	6.15±0.19 a	5.65±0.21 ab	4.79±0.15 b	6.46±0.28 a	5.66±0.65 ab	6.26±0.10 a	*
ethyl 9-decenoate		5.41±0.33	5.01±0.52	4.89±0.19	4.58±0.29	5.32±0.08	5.18±0.14	5.14±0.07	NS
benzeneacetic acid, ethyl ester		5.28±0.09 ab	4.90±0.13 c	4.91±0.00 c	4.95±0.00 bc	5.21±0.05 abc	5.03±0.09 abc	5.39±0.10 ab	**
acetic acid, 2-phenylethyl ester		6.09±0.01 bc	5.74±0.00 c	6.66±0.07 a	6.61±0.13 a	6.16±0.14 bc	6.37±0.19 abc	6.59±0.14 a	**
dodecanoic acid, ethyl ester		5.92±0.09 ab	5.72±0.18 ab	5.49±0.02 ab	5.15±0.01 b	6.09±0.39 a	5.67±0.37 ab	6.04±0.04 a	*
tetradecanoic acid, ethyl ester		5.21±0.19 ab	4.72±0.17 b	5.01±0.06 ab	5.28±0.05 ab	5.40±0.10 ab	5.39±0.30 ab	5.50±0.15 a	*
pentadecanoic acid, ethyl ester		4.93±0.35	4.32±0.33	4.25±0.36	4.56±0.36	4.99±0.24	4.95±0.03	4.92±0.26	NS
ethyl anisate		5.29±0.11 a	4.74±0.06 b	4.84±0.08 b	5.14±0.13 ab	5.44±0.02 a	5.23±0.15 a	5.48±0.12 a	**
ethyl 4-methoxyphenylacetate	X	5.88±0.01 ab	5.43±0.13 b	5.45±0.18 b	5.70±0.22 ab	6.21±0.08 a	5.92±0.12 ab	6.02±0.19 ab	**
hexadecanoic acid, ethyl ester	X	5.27±0.23 abc	4.82±0.07 c	4.77±0.13 c	5.03±0.12 bc	5.61±0.10 ab	5.43±0.15 ab	5.63±0.09 ab	**

(Continued)

Compound	Must	*SC*	*PJ*	*TD*	*KL*	*SC + PJ*	*SC + TD*	*SC + KL*	ANO-VA
Esters (continued)									
ethyl 9-hexadecenoate		5.08±0.19 a	nd	4.58±0.19 a	4.74±0.09 a	nd	5.07±0.37 a	5.25±0.18 a	***
decanedioic acid, diethyl ester		4.89±0.01 abc	4.39±0.13 cd	4.25±0.22 d	4.47±0.14 cd	5.21±0.17 ab	4.98±0.15 ab	5.17±0.01 ab	**
2-decenedioic acid, diethyl ester		4.75±0.03 a	nd	nd	4.39±0.41 a	5.02±0.11 a	4.78±0.32 a	4.90±0.09 a	***
Lactones									
γ-nonalactone		4.92±0.09 ab	4.43±0.14 c	4.60±0.17 abc	4.72±0.11 abc	5.08±0.11 ab	4.81±0.03 abc	4.99±0.08 ab	**
γ-decalactone		4.76±0.09 ab	4.31±0.10 c	4.44±0.14 bc	4.60±0.05 abc	4.96±0.11 ab	4.61±0.05 abc	4.88±0.17 ab	**
Terpenoids									
trans-linalool oxide	X	5.32±0.07 a	5.09±0.03 ab	4.91±0.01 b	5.09±0.10 ab	5.36±0.05 a	5.14±0.16 ab	5.40±0.00 a	**
cis-linalool oxide	X	4.66±0.18 a	4.35±0.06 a	nd	4.48±0.04 a	4.68±0.10 a	nd	4.72±0.07 a	***
ß -linalool	X	5.01±0.01	4.90±0.10	4.95±0.12	4.99±0.09	5.00±0.10	4.98±0.13	5.11±0.01	NS
hotrienol	X	5.25±0.10	5.20±0.05	5.04±0.08	5.11±0.16	5.20±0.52	5.30±0.19	5.43±0.09	NS
epoxylinalol	X	4.88±0.04 ab	4.45±0.06 b	4.67±0.20 ab	4.69±0.17 ab	5.00±0.07 a	4.78±0.04 ab	4.94±0.10 a	*
Others									
ß-damascenone		4.81±0.11 a	4.61±0.04 a	4.67±0.07 a	4.61±0.26 a	nd	nd	5.00±0.13 a	***
acetoin		6.03±0.00 a	nd	nd	nd	5.33±0.28 a	5.36±0.38 a	5.87±0.42 a	***
1-propanol, 3-ethoxy		nd	nd	4.40±0.39 a	4.56±0.09 a	nd	4.67±0.08 a	4.70±0.04 a	***
benzaldehyde	X	5.66±0.06	5.49±0.44	5.44±0.15	5.28±0.67	5.29±0.25	5.98±0.12	5.63±0.20	NS
p-anisaldehyde	X	5.76±0.09	5.04±0.19	5.20±0.28	5.51±0.20	5.77±0.02	5.72±0.23	5.80±0.21	*

*X = compound is present in must; nd = not detected; ANOVA: NS = not significant. * = P < 0.05. ** = P < 0.01. *** = P < 0.001; different letters in same row mean samples are significantly different according to Tukey's HSD post-hoc test (P<0.05)*

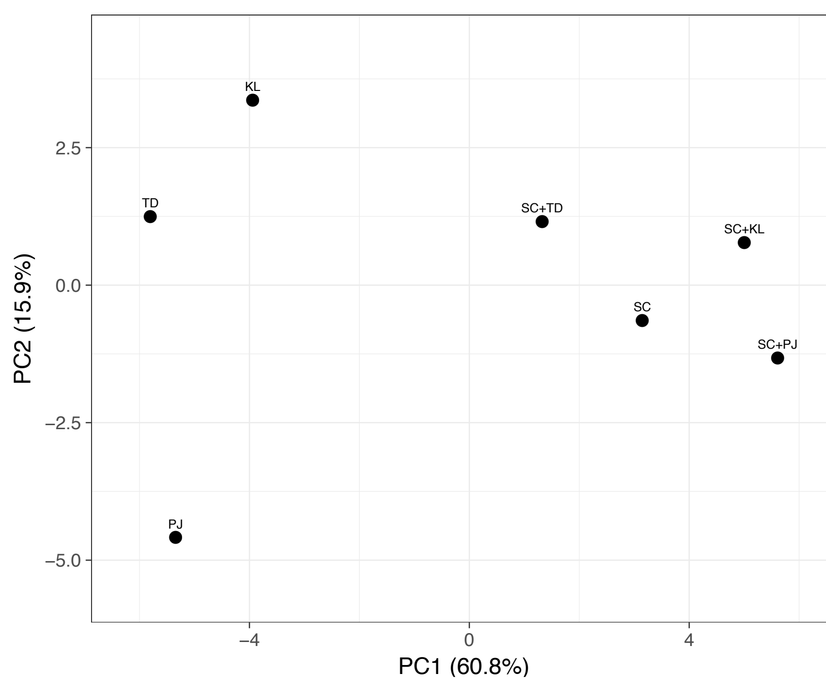

Figure 1. Principal component analysis of mead samples based on logarithmic peak areas of volatile organic compounds detected by GC-MS. SC = *Saccharomyces cerevisiae*, PJ = *Pichia jadinii*, TD = *Torulaspora delbrueckii*, KL = *Kluyveromyces lactis.*

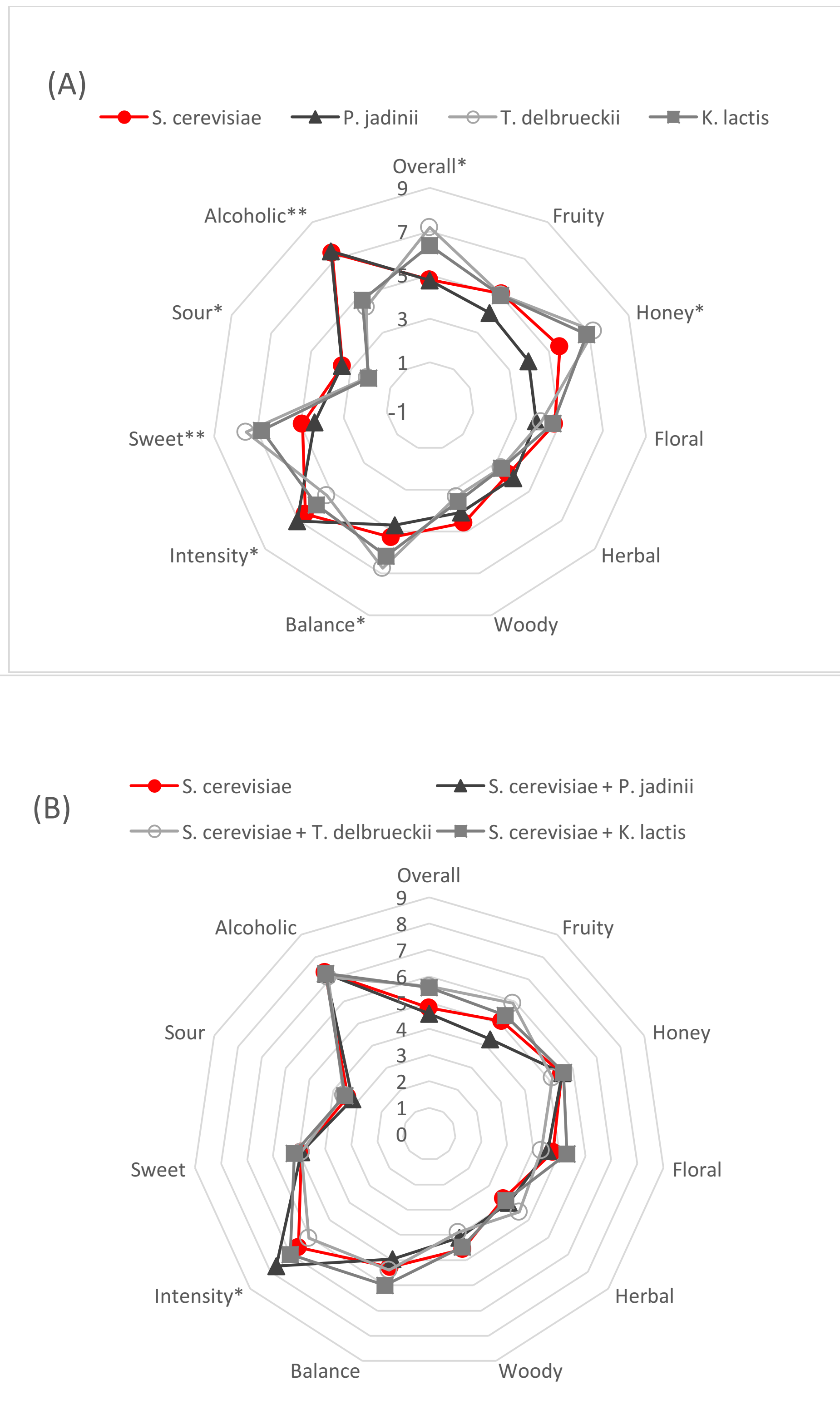

Figure 2. Results of sensory analysis including overall appreciation and intensity of taste and flavor attributes (scale 1-9) as judged by panel (n=21), comparing meads produced with single strain *S. cerevisiae* with other single culture inoculations (3A) and mixed culture inoculations (3B). Stars next to attribute labels indicate significance level according to ANOVA (*: P <0.01, **: P<0.001).

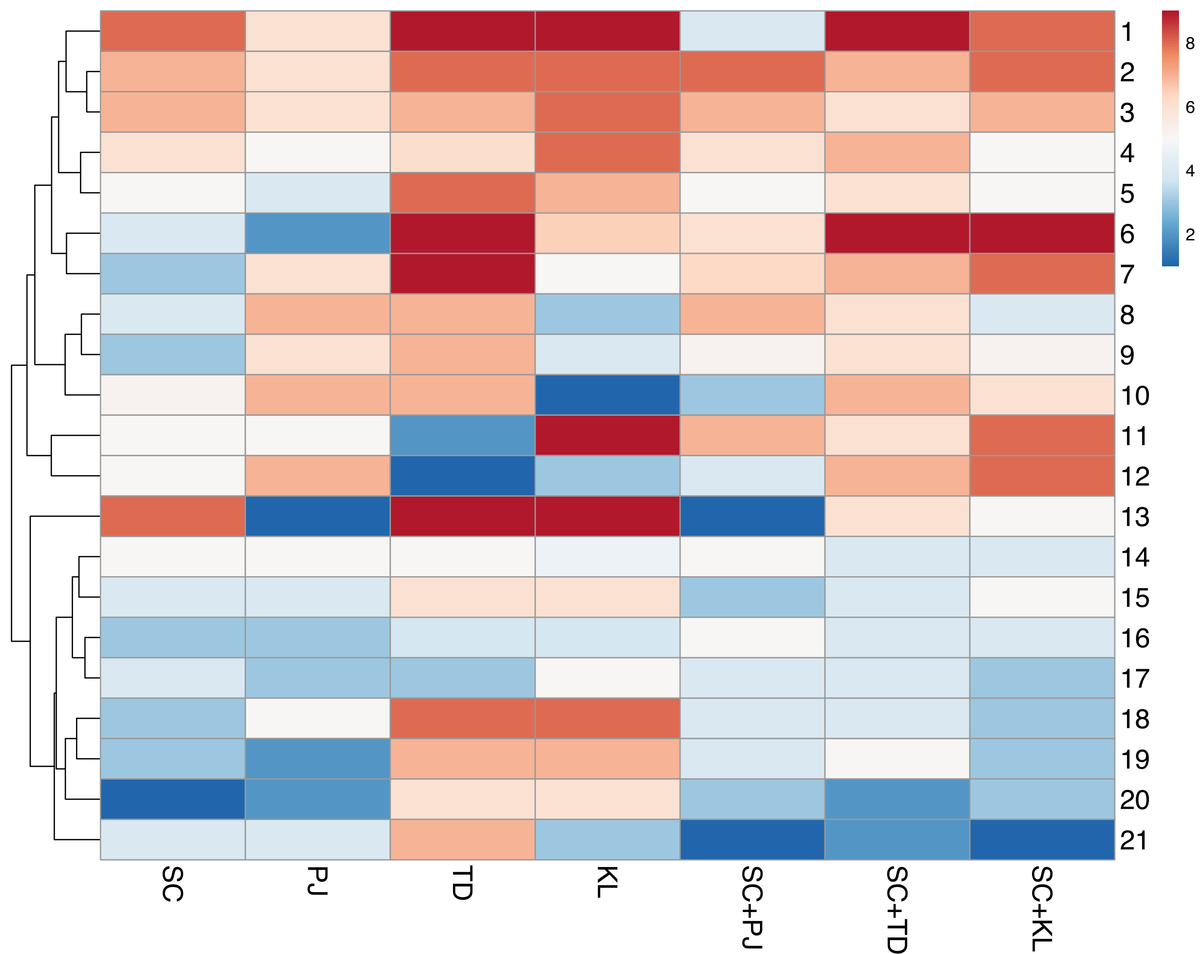

Figure 3. Heatmap recording overall appreciation (hedonic scale, 1-9) for mead samples expressed during the sensory analysis by individual panelists (numbered 1-21). Panelists are clustered by Euclidian distance. SC = *Saccharomyces cerevisiae*, PJ = *Pichia jadinii*, TD = *Torulaspora delbrueckii*, KL = *Kluyveromyces lactis.*

4 Conclusions

The results presented in this paper demonstrate that *T. delbrueckii* and *K. lactis* are promising candidate starter cultures for the production of low-alcoholic meads. Meads produced by these yeasts, alone or in co-culture with *S. cerevisiae*, had a higher acceptance rate than meads produced by *S. cerevisiae* on its own. Both strains produced significantly higher amounts of honey-aroma compound phenethyl acetate in both single strain and mixed fermentations. Using mixed culture fermentations can therefore contribute to the complexity of the aroma profiles of meads. Moreover, sensory panelists in this study expressed a preference for low-alcoholic meads with high residual sweetness, a product category that warrants further exploration.

5 References

ABEMEL, 2018. Dados das exportações de mel [WWW Document]. URL http://brazilletsbee.com.br/dados-setoriais.aspx (accessed 2.11.19).

Anupama, D., Bhat, K.., Sapna, V.., 2003. Sensory and physico-chemical properties of commercial samples of honey. Food Res. Int. 36, 183–191. https://doi.org/10.1016/S0963-9969(02)00135-7

Bartowsky, E.J., Pretorius, I.S., 2009. Microbial Formation and Modification of Flavor and Off-Flavor Compounds in Wine, in: Biology of Microorganisms on Grapes, in Must and in Wine. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 209–231. https://doi.org/10.1007/978-3-540-85463-0_11

Basso, R.F., Alcarde, A.R., Portugal, C.B., 2016. Could non-Saccharomyces yeasts contribute on innovative brewing fermentations? Food Res. Int. 86, 112–120. https://doi.org/10.1016/J.FOODRES.2016.06.002

Bastos, E.M.A.F., de Souza São Thiago Calaça, P., Simeão, C.M.G., Rodrigues Ribeiro da Cunha, M., 2016. Characterization of the honey from Myracrodruon urundeuva (Anacardiceae-Aroeira) in the Dry Forest of northern of Minas Gerais/Brazil. Adv. Agric. Sci. 4, 64–71.

Bressani, A.P.P., Martinez, S.J., Evangelista, S.R., Dias, D.R., Schwan, R.F., 2018. Characteristics of fermented coffee inoculated with yeast starter cultures using different inoculation methods. LWT 92, 212–219. https://doi.org/10.1016/J.LWT.2018.02.029

Chen, D., Liu, S.-Q., 2016. Impact of simultaneous and sequential fermentation with Torulaspora delbrueckii and Saccharomyces cerevisiae on non-volatiles and volatiles of lychee wines. LWT - Food Sci. Technol. 65, 53–61. https://doi.org/10.1016/J.LWT.2015.07.050

Ciani, M., Comitini, F., 2015. Yeast interactions in multi-starter wine fermentation. Curr. Opin. Food Sci. 1, 1–6. https://doi.org/10.1016/J.COFS.2014.07.001

Dashko, S., Zhou, N., Tinta, T., Sivilotti, P., Lemut, M.S., Trost, K., Gamero, A., Boekhout, T., Butinar, L., Vrhovsek, U., Piskur, J., 2015. Use of non-conventional yeast improves the wine aroma profile of Ribolla Gialla. J. Ind. Microbiol. Biotechnol. 42, 997–1010. https://doi.org/10.1007/s10295-015-1620-y

Ducki, S., Miralles-Garcia, J., Zumbé, A., Tornero, A., Storey, D.M., 2008. Evaluation of solid-phase micro-extraction coupled to gas chromatography–mass spectrometry for the headspace analysis of volatile compounds in cocoa products. Talanta 74, 1166–1174. https://doi.org/10.1016/J.TALANTA.2007.08.034

Evangelista, S.R., da Cruz Pedrozo Miguel, M.G., de Souza Cordeiro, C., Silva, C.F., Marques Pinheiro, A.C., Schwan, R.F., 2014. Inoculation of starter cultures in a semi-dry coffee (Coffea arabica) fermentation process. Food Microbiol. 44, 87–95. https://doi.org/10.1016/j.fm.2014.05.013

Gamero, A., Quintilla, R., Groenewald, M., Alkema, W., Boekhout, T., Hazelwood, L., 2016. High-throughput screening of a large collection of non-conventional yeasts reveals their potential for aroma formation in food fermentation. Food Microbiol. 60, 147–159. https://doi.org/10.1016/J.FM.2016.07.006

Garg, N., Sethupathy, A., Tuwani, R., NK, R., Dokania, S., Iyer, A., Gupta, A., Agrawal, S., Singh, N., Shukla, S., Kathuria, K., Badhwar, R., Kanji, R., Jain, A., Kaur, A., Nagpal, R., Bagler, G., 2018. FlavorDB: a database of flavor molecules. Nucleic Acids Res. 46, D1210–D1216. https://doi.org/10.1093/nar/gkx957

Gomes, T., Dias, T., Cadavez, V., Verdial, J., Morais, J.S., Ramalhosa, E., Estevinho, L.M., 2015. Influence of sweetness and ethanol content on mead acceptability. Polish J. Food Nutr. Sci. 65, 137–142. https://doi.org/10.1515/pjfns-2015-0006

Gutiérrez, A., Boekhout, T., Gojkovic, Z., Katz, M., 2018. Evaluation of non- *Saccharomyces* yeasts in the fermentation of wine, beer and cider for the development of new beverages. J. Inst. Brew. 124, 389–402.https://doi.org/10.1002/jib.512

Hopfer, H., Heymann, H., 2014. Judging wine quality: Do we need experts, consumers or trained panelists? Food Qual. Prefer. 32, 221–233. https://doi.org/10.1016/J.FOODQUAL.2013.10.004

Jolly, N.P., Augustyn, O.P.H., Pretorius, I.S., 2017. The Role and Use of Non-Saccharomyces Yeasts in Wine Production. South African J. Enol. Vitic. 27. https://doi.org/10.21548/27-1-1475

Lilly, M., Bauer, F.F., Lambrechts, M.G., Swiegers, J.H., Cozzolino, D., Pretorius, I.S., 2006. The effect of increased yeast alcohol acetyltransferase and esterase activity on the flavour profiles of wine and distillates. Yeast 23, 641–659. https://doi.org/10.1002/yea.1382

Loira, I., Vejarano, R., Bañuelos, M.A., Morata, A., Tesfaye, W., Uthurry, C., Villa, A., Cintora, I., Suárez-Lepe, J.A., 2014. Influence of sequential fermentation with Torulaspora delbrueckii and Saccharomyces cerevisiae on wine quality. LWT - Food Sci. Technol. 59, 915–922. https://doi.org/10.1016/J.LWT.2014.06.019

Maturano, Y.P., Rodríguez Assaf, L.A., Toro, M.E., Nally, M.C., Vallejo, M., Castellanos de Figueroa, L.I., Combina, M., Vazquez, F., 2012. Multi-enzyme production by pure and mixed cultures of Saccharomyces and non-Saccharomyces yeasts during wine fermentation. Int. J. Food Microbiol. 155, 43–50. https://doi.org/10.1016/J.IJFOODMICRO.2012.01.015

Mestre Furlani, M.V., Maturano, Y.P., Combina, M., Mercado, L.A., Toro, M.E., Vazquez, F., 2017. Selection of non-Saccharomyces yeasts to be used in grape musts with high alcoholic potential: a strategy to obtain wines with reduced ethanol content. FEMS Yeast Res. 17. https://doi.org/10.1093/femsyr/fox010

Metsalu, T., Vilo, J., 2015. ClustVis: a web tool for visualizing clustering of multivariate data using Principal Component Analysis and heatmap. Nucleic Acids Res. 43, W566–W570. https://doi.org/10.1093/nar/gkv468

Mendes-Ferreira, A., Cosme, F., Barbosa, C., Falco, V., Inês, A., Mendes-Faia, A., 2010. Optimization of honey-must preparation and alcoholic fermentation by Saccharomyces cerevisiae for mead production. Int. J. Food Microbiol. 144, 193–198. https://doi.org/10.1016/J.IJFOODMICRO.2010.09.016

Pereira, A.P., Mendes-Ferreira, A., Oliveira, J.M., Estevinho, L.M., Mendes-Faia, A., 2015. Mead production: Effect of nitrogen supplementation on growth, fermentation profile and aroma formation by yeasts in mead fermentation. J. Inst. Brew. 121, 122–128. https://doi.org/10.1002/jib.184

Pereira, A.P., Mendes-Ferreira, A., Estevinho, L.M., Mendes-Faia, A., 2015. Improvement of mead fermentation by honey-must supplementation. J. Inst. Brew. 121, 405–410. https://doi.org/10.1002/jib.239

Pereira, A.P., Mendes-Ferreira, A., Oliveira, J.M., Estevinho, L.M., Mendes-Faia, A., 2013. High-cell-density fermentation of Saccharomyces cerevisiae for the optimisation of mead production. Food Microbiol. 33, 114–123. https://doi.org/10.1016/J.FM.2012.09.006

Peryam, D., Pilgrim, F., 1957. Hedonic scale method of measuring food preferences. Food Technol. 11, 9–14.

Quirós, M., Rojas, V., Gonzalez, R., Morales, P., 2014. Selection of non-Saccharomyces yeast strains for reducing alcohol levels in wine by sugar respiration. Int. J. Food Microbiol. 181, 85–91. https://doi.org/10.1016/J.IJFOODMICRO.2014.04.024

Ramalhosa, E., Gomes, T., Pereira, A.P., Dias, T., Estevinho, L.M., 2011. Mead Production: Tradition Versus Modernity. Adv. Food Nutr. Res. 63, 101–118. https://doi.org/10.1016/B978-0-12-384927-4.00004-X

Ribeiro, L.S., Miguel, M.G. da C.P., Evangelista, S.R., Martins, P.M.M., van Mullem, J., Belizario, M.H., Schwan, R.F., 2017. Behavior of yeast inoculated during semi-dry coffee fermentation and the effect on chemical and sensorial properties of the final beverage. Food Res. Int. 92, 26–32. https://doi.org/10.1016/J.FOODRES.2016.12.011

Ruyters, S., Mukherjee, V., Verstrepen, K.J., Thevelein, J.M., Willems, K.A., Lievens, B., 2015. Assessing the potential of wild yeasts for bioethanol production. J. Ind. Microbiol. Biotechnol. 42, 39–48. https://doi.org/10.1007/s10295-014-1544-y

Saerens, S.M.G., Delvaux, F.R., Verstrepen, K.J., Thevelein, J.M., 2010. Production and biological function of volatile esters in *Saccharomyces cerevisiae*. Microb. Biotechnol. 3, 165–177. https://doi.org/10.1111/j.1751-7915.2009.00106.x

Šmogrovičová, D., Nádaský, P., Lich, R.T., Wilhelmi, B.S., Cambray, G., 2012. Analytical and aroma profiles of Slovak and South African meads. Czech J. Food Sci. 30, 241–246. https://doi.org/10.1080/13669870903136175

Sroka, P., Tuszyński, T., 2007. Changes in organic acid contents during mead wort fermentation. Food Chem. 104, 1250–1257. https://doi.org/10.1016/j.foodchem.2007.01.046

Steinkraus, K.H., Morse, R.A., 1973. Chemical Analysis of Honey Wines. J. Apic. Res. 12, 191–195. https://doi.org/10.1080/00218839.1973.11099749

Tronchoni, J., Gamero, A., Arroyo-López, F.N., Barrio, E., Querol, A., 2009. Differences in the glucose and fructose consumption profiles in diverse Saccharomyces wine species and their hybrids during grape juice fermentation. Int. J. Food Microbiol. 134, 237–243. https://doi.org/10.1016/J.IJFOODMICRO.2009.07.004

Varela, C., Sengler, F., Solomon, M., Curtin, C., 2016. Volatile flavour profile of reduced alcohol wines fermented with the non-conventional yeast species Metschnikowia pulcherrima and Saccharomyces uvarum. Food Chem. 209, 57–64. https://doi.org/10.1016/J.FOODCHEM.2016.04.024

Viana, F., Gil, J. V., Genovés, S., Vallés, S., Manzanares, P., 2008. Rational selection of non-Saccharomyces wine yeasts for mixed starters based on ester formation and enological traits. Food Microbiol. 25, 778–785. https://doi.org/10.1016/J.FM.2008.04.015

Vidrih, R., Hribar, J., 2016. Mead: The Oldest Alcoholic Beverage, in: Traditional Foods. Springer US, Boston, MA, pp. 325–338. https://doi.org/10.1007/978-1-4899-7648-2_26

Vidrih, R., Hribar, J., 2007. Studies on the sensory properties of mead and the formation of aroma compounds related to the type of honey. Acta Aliment. 36, 151–162. https://doi.org/10.1556/AAlim.36.2007.2.2

Zara, G., Mannazzu, I., Del Caro, A., Budroni, M., Pinna, M.B., Murru, M., Farris, G.A.,

Zara, S., 2014. Wine quality improvement through the combined utilisation of yeast hulls and *C andida zemplinina* / *S accharomyces cerevisiae* mixed starter cultures. Aust. J. Grape Wine Res. 20, 199–207. https://doi.org/10.1111/ajgw.12078

Zha, Y., Hossain, A.H., Tobola, F., Sedee, N., Havekes, M., Punt, P.J., 2013. *Pichia anomala* 29X: a resistant strain for lignocellulosic biomass hydrolysate fermentation. FEMS Yeast Res. 13, 609–617. https://doi.org/10.1111/1567-1364.12062

Appendix 1

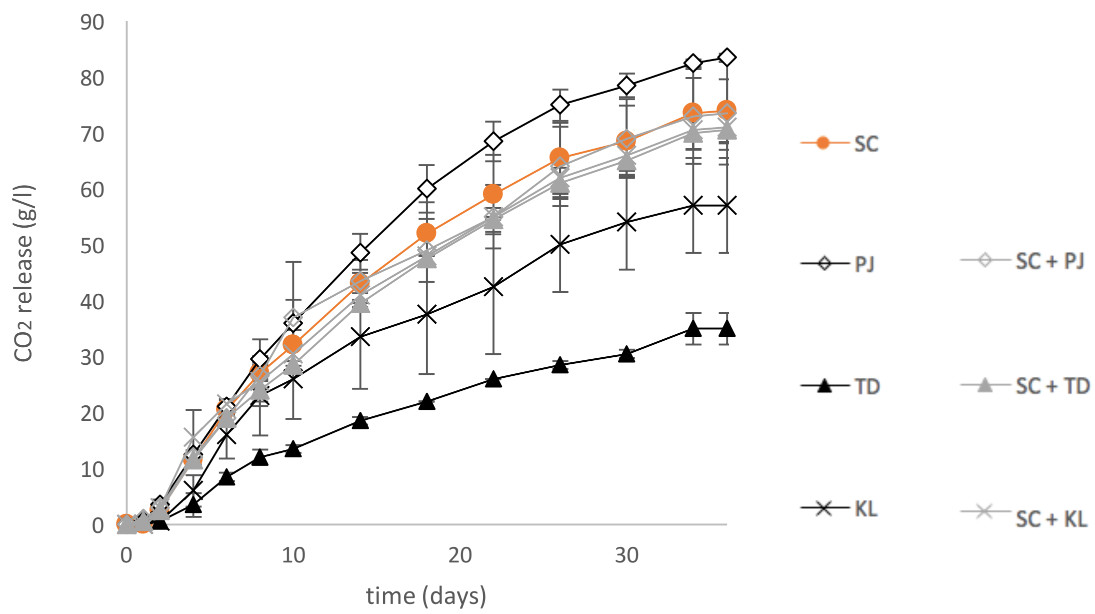

Figure 1. Release of carbon dioxide during mead fermentations. SC = *Saccharomyces cerevisiae*, PJ = *Pichia jadinii*, TD = *Torulaspora delbrueckii*, KL = *Kluyveromyces lactis*.

Appendix 2

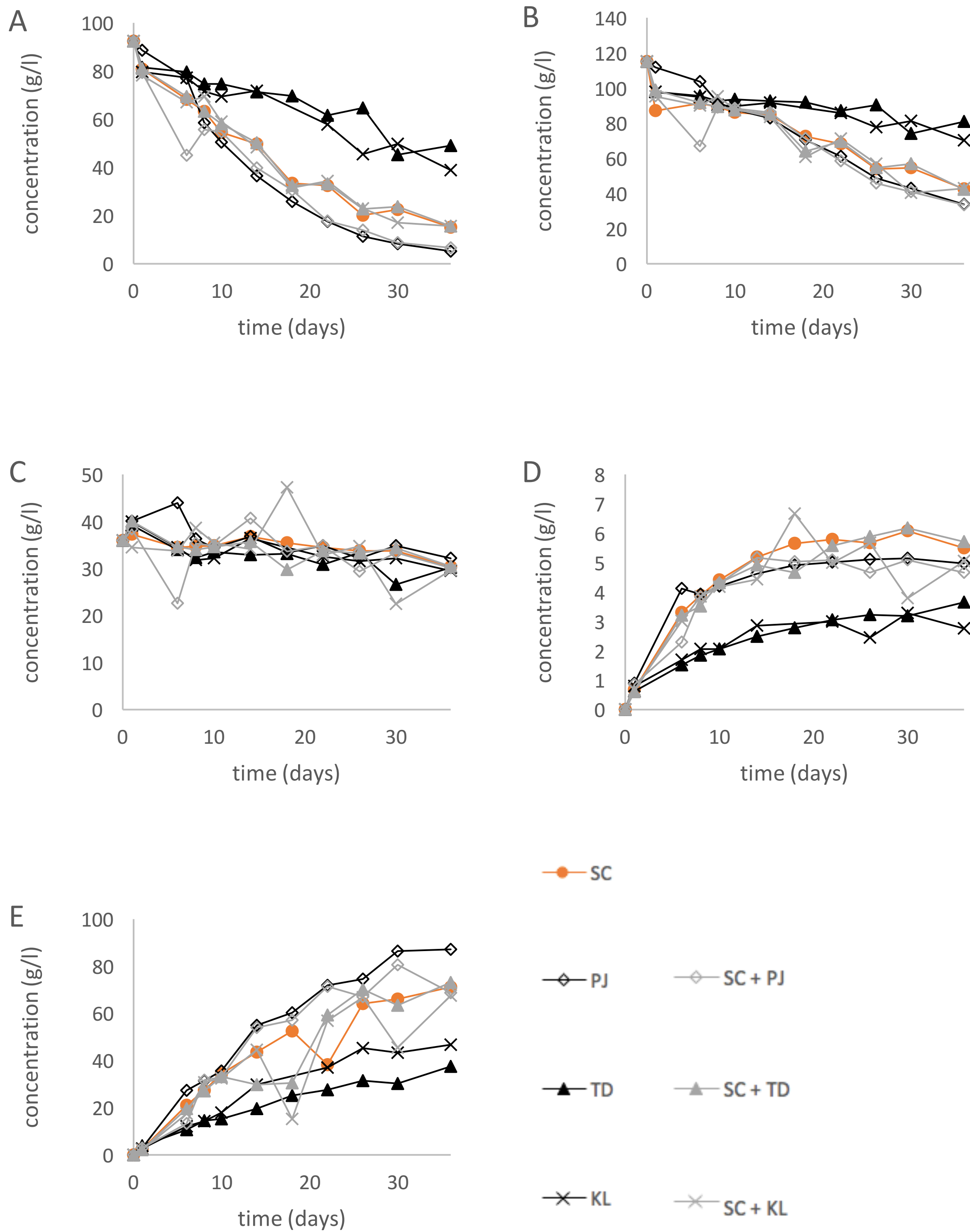

Figure 1a. Evolution of concentrations of glucose (A), fructose (B), maltose (C), glycerol (D) and ethanol (E) during mead fermentations. SC = *Saccharomyces cerevisiae*, PJ = *Pichia jadinii*, TD = *Torulaspora delbrueckii*, KL = *Kluyveromyces lactis*.

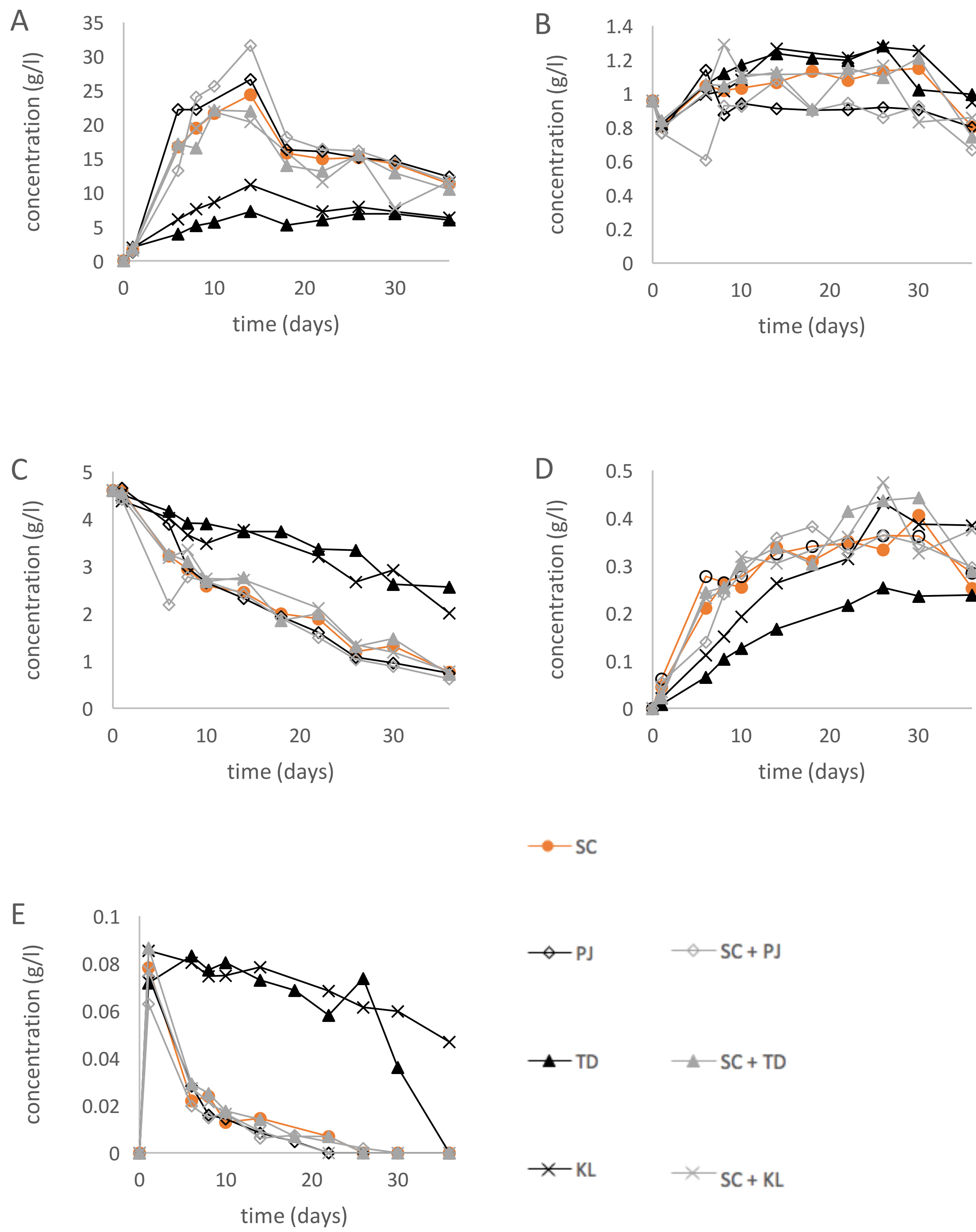

Figure 1b. Evolution of concentrations of citric acid (A), succinic acid (B), malic acid (C), acetic acid (D) and oxalic acid (E) during mead fermentations. SC = *Saccharomyces cerevisiae*, PJ = *Pichia jadinii*, TD = *Torulaspora delbrueckii*, KL = *Kluyveromyces lactis*.

Appendix 3

Table 1. Odor descriptors of volatile organic compounds encountered in meads

Compound	descriptors
1-butanol, 3-methyl	oil, alcoholic, burnt, whiskey, malt, banana, fusel, fruity
2,3-butanediol	onion, fruit, creamy, fruity, buttery
benzyl alcohol	berry, balsamic, rose, floral, walnut, sweet, cherry, phenolic, flower, grapefruit
phenethyl alcohol	lilac, rose, rose water, honey, rose flower, floral, spice, bitter, rose dried
p-anisylalcohol	caramel, chocolate, powdery, vanilla, rose, lilac, floral, sweet, honey, hawthorn, hyacinth, flower
2-(4-methoxyphenyl)-ethanol	unknown
butanoic acid, 3-methyl	sour, sweat, acid, stinky, sweaty, animal, rancid, tropical, feet, cheese
benzeneacetic acid, 4-methoxy	unknown
octanoic acid	cheesy, sweat, vegetable, waxy, fatty, rancid, oily, cheese
nonanoic acid	cultured dairy, fat, waxy, green, dirty, cheese
n-decanoic acid	sour, citrus, fat, rancid, fatty, unpleasant
benzoic acid	balsam, urine, faint
dodecanoic acid	coconut, mild, fatty, metal, bay oil
tetradecanoic acid	fatty, soapy, waxy, coconut
octanoic acid, ethyl ester	apricot, fat, wine, waxy, banana, brandy, fruit, sweet, fruity, pear
decanoic acid, ethyl ester	apple, brandy, waxy, grape, oily, sweet, fruity, pear
ethyl 9-decenoate	unknown

Compound	descriptors
benzeneacetic acid, ethyl ester	grapefruit, balsam, rose, anise, chocolate, honey, melon, fruit, sweet, floral, raspberry, bitter, cocoa
Phenethyl acetate	tobacco, rose, floral, sweet, honey, fruity, tropical
dodecanoic acid, ethyl ester	waxy, floral, sweet, clean, leaf, soapy
tetradecanoic acid, ethyl ester	ether, waxy, sweet, soapy, orris, violet
pentadecanoic acid, ethyl ester	unknown
ethyl anisate	aniseed, anise, fruity, balsam, tarragon
ethyl 4-methoxyphenylacetate	unknown
hexadecanoic acid, ethyl ester	mild, wax, milky, waxy, creamy, fruity, balsam
ethyl 9-hexadecenoate	unknown
decanedioic acid, diethyl ester	unknown
2-decenedioic acid, diethyl ester	unknown
gamma nonalactone	peach, coconut, waxy, oily, creamy, sweet, buttery
gamma decalactone	peach, coconut, caramel, fat, waxy, fresh, oily, fatty, sweet, fruity, strong, buttery
trans-linalool oxide	floral, flower
cis-linalool oxide	fresh, sweet, pine, floral
beta-linalool	lemon, citrus, orange, floral, sweet, woody, blueberry, bois de rose, lavender, flower, green
hotrienol	mouldy
epoxylinalol	musty, alcohol, fenchyl, camphor
beta-damascenone	apple, tobacco, berry, blackcurrant, rose, floral, plum, fruity, honey

Compound	descriptors
acetoin	butter, cream, milky, fatty, creamy, sweet, dairy, buttery
1-propanol, 3-ethoxy	fruit
benzaldehyde	cherry, almond, sweet, burnt sugar, sharp, strong, bitter
p-anisaldehyde	mimosa, hawthorn, cherry, powdery, bitter, vanilla, anise, hawthorne, chocolate, balsam, creamy, minty, berry, sweet, floral, mint, almond, cinnamon

Source: FlavorDB (https://cosylab.iiitd.edu.in/flavordb/)