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LIPIDOMIC AND METABOLOMIC TOOLS FROM COLONY FORMING UNITS OF WINE MICROORGANISMS ON AGAR PLATES

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Lipidomic and metabolomic tools from Colony Forming Units of wine microorganisms on agar plates

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Abstract

Winemaking is a biotechnological process in which many microorganisms such as yeasts and lactic acid bacteria (LAB) are involved. However, some of them are undesirable, as molds, acetic acid bacteria (AAB) and some species of yeast and LAB. Studies of ecological and physiological presence of these microorganisms in this process depend mainly on the cultivable quality of them. In a general way and firstly, the isolation of these microorganisms on solid selective growth media according to the species is used. Then, the behaviour of each species is characterised on liquid growth media.

The aim of this work is to study the metabolomic and lipidomic profiles of colony-forming units (CFU) of some wine species growing on solid selective or universal media. The effects of growth time, number of CFUs and different growing conditions will be studied. We observed significant changes, one of the most relevant were the variations of lipid composition and metabolome over time. The differences between fatty acid composition of yeast, LAB and AAB species grown on a universal culture media were also remarkable. The results suggest that the method of gas chromatography used, and the discriminant model created could could be applied for identification and metabolic characterization of oenological microorganisms.

Keywords: *yeast*, lactic acid bacteria, acetic acid bacteria, lipid composition, metabolome, identification.

Resumen

La vinificación es un proceso biotecnológico en el que participan diversos microorganismos, principalmente levaduras y bacterias lácticas. Sin embargo, no todas las especies son de interés, además encontramos otros microrganismos indeseables como hongos y bacterias acéticas. Dada su importancia en el vino se realizan numerosos estudios ecológicos y fisiológicos de estos microorganismos, siendo la mayoría aislados en medios de cultivo sólido selectivos para el tipo de microorganismo a identificar. Posteriormente se estudia su comportamiento en medios de cultivo líquidos.

El objetivo de este trabajo es estudiar los perfiles metabolómicos y lipidómicos de unidades formadoras de colonia de algunas especies vínicas, tanto beneficiosas como indeseables, crecidas en medios de cultivo selectivos y universales. Se estudiarán los efectos del tiempo, número de UFCs y diferentes condiciones de crecimiento en levaduras, además de comparar la composición en ácidos grasos de los diferentes microorganismos. Con respecto a los resultados se observaron cambios significativos, siendo uno de los más relevantes la variación de los perfiles lipidómicos y metabolómicos en función del tiempo indiferentemente del número de UFCs. Por otra parte, se consiguieron diferenciar muchas de las especies analizadas por su perfil de ácidos grasos, observando diferencias entre levaduras, BAL y BAA crecidas en un mismo medio de cultivo universal. Los resultados apuntan a que la utilización de la cromatografía de gases sumada al modelo discriminante creado podría ser aplicado para la caracterización metabólica e identificación de microrganismos vínicos.

Palabras clave: ***levaduras, bacterias lácticas, bacterias acéticas, composición lipídica, metaboloma, identificación.***

1. Introduction

1.1. Importance of microbiology in winemaking

From vineyards to cellars, different types of microorganisms are present and play an important role in wine elaboration. Yeasts (*Saccharomyces* and non-*Saccharomyces* species) and lactic acid bacteria (LAB) are the main protagonists of winemaking, being the responsible of alcoholic and malolactic fermentations, respectively (Fugelsang & Edwards 2007). Nevertheless, other undesirable microorganisms like acetic acid bacteria (AAB) and molds are also found. All this microbiota can work at different levels, in vine healthy, cellar contamination, typicity or spoilage of wines, among others (Fugelsang & Edwards 2007).

Therefore, one of the main responsibilities of the winemaker should be an exhaustive microbiological control. Firstly, avoiding displeasing microorganisms with a thorough cleaning of the cellar, followed by the prevention of grapevine diseases and the preservation of grape's microbiota with the right viticulture practices (Pretorius 2000). Grapevine conditions are important due to the presence of many microorganisms on the surface of grapes. Some of them are interesting from a winemaking point of view, as some non-*Saccharomyces* yeasts. These yeast have been traditionally considered undesired microorganisms because of their low fermentation capacity (Manzanares et al. 2011). Secondly, another important point is the way to carry out the alcoholic and malolactic fermentations, which can be performed with autochthonous or commercial microorganisms. On the one hand, some wineries prefer a spontaneous fermentation with natural microbiota because it can provide unique organoleptic characteristics in wine, typical of the area and vintage. However, spontaneous fermentations present a certain risk of stuck or sluggish fermentations in addition to the lack of reproducibility year after year (Pretorius 2000). On the other hand, another possibility is the use of starter cultures, which control the fermentative process, but limiting wine typicity. In this way, the use of non-*Saccharomyces* together with *S. cerevisiae* in mixed starters cultures and the selection of indigenous yeasts are increasing. As a result, the organoleptic complexity of wine is improved, combining advantages of both strategies (Tempère et al. 2018).

1.2. Microbiological control and culture media

Wine microorganisms are subject of selection and characterisation in many studies due to their relevance in winemaking (Padilla et al. 2016; Portillo et al. 2016; Franquès et al. 2017). In order to assess the suitability of microorganisms involved in the process, they must be first isolated and then, identified. For isolation, it is necessary to use a specific culture media, depending on the microorganism physiology. There are non-selective media, like Man Rogosa Sharpe (MRS) medium (Man et al. 1960) which is the most common medium to isolate LAB, and it can be supplemented with grape juice, tomato, cysteine, malic acid, and several sugars (Muñoz et al. 2011). In addition, cycloheximide and pimaricin can be also added to inhibit the growth of yeast and fungi (Muñoz et al. 2011). Regarding yeasts, the commonly used culture media are rich

media such as yeast extract peptone dextrose agar medium (YPDA). YPDA contains an energy source (e.g., glucose, fructose, or sucrose), a hydrolysed protein a complex vitamin supplement as yeast, meat or malt extract. The media can be also supplemented with antibiotics to prevent bacterial growth or antifungals to inhibit fungal growth (Manzanares et al. 2011). In the case of AAB, the culture media have a carbon source as glucose, mannitol or ethanol and can also be incorporated $CaCO_3$ or bromocresol-green as acid indicators. The most used media are glucose yeast extract $CaCO_3$ medium (GYC) and yeast extract peptone mannitol medium (YPM).These media are usually supplemented with pimaricin or similar antifungals to avoid yeasts and molds growth or with penicillin to avoid Gram positive bacteria as LAB (Guilamón & Mas, 2009.).

In oenology, the use of selective media is interesting not only for differentiating *S. cerevisiae* from non-*Saccharomyces* yeasts, but also for detecting *Saccharomyces* spoilage yeasts. In this case, the best option is the lysine agar medium, which allows *S. cerevisiae* to be distinguished from non-*Saccharomyces* yeasts, as a consequence of the inability of *S.cerevisiae* to use lysine as a nitrogen source (Manzanares et al. 2011). Furthermore, there is another type of culture media, the differential ones. One example is Wallersteins Nutrient (WL) agar, which allows identifying yeast by colony morphology and dye uptake (Manzanares et al. 2011). In addition, the use of medium containing bismuth sulphite (BIGGY agar) is a good indicator of yeast producing H_2S (Jiranek et al. 1995).

These culture media have been used during many years for identifying and characterising microorganisms regarding to their phenotypic characteristics. Despite the fact that, in recent years, they have been almost supplanted by molecular techniques, they are still used in oenology.

Molecular biology -based methods are effective for both yeasts and bacteria. Many methods have been described: quantitative real-time polymerase chain reaction (q-PCR); sequencing methods as multilocus sequence typing or whole genome sequencing and fingerprinting methods as restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLP) or microsatellites (Ivey & Phister 2011). Other technique, which is less studied, is the gas-liquid chromatography of long-chain fatty acids, resulting on fingerprinting peaks on chromatograms (Pretorius et al. 1999).

1.3. Identification and characterization of microorganisms by total cellular fatty acid profiles

Chemotaxonomic analysis involves methods that differentiate and classify microorganisms by determining chemical markers such as lipids, proteins or other cell constituents. Since Abel et al. demonstrated in 1963 that microorganisms can be classified into genera and species by their lipid composition by using gas chromatography, this technique has been used in many studies to differentiate yeast species by their lipids fingerprint. Gunasekaran & Hughes (1980) developed a rapid method to identify several species of *Candida,* based on the presence or absence of certain fatty-acids in their lipid composition and, Kock et al. (1985), applied the same method to identify four species of *Endomycetaceae*.

1.3.1.Lipid yeast composition.

Profiles of cellular fatty acids have been also applied in oenological yeasts. Rozès et al. (1992) achieved the differentiation of *S. cerevisiae* from other spoilage yeasts by their total fatty acid composition. However, this technique presented some disadvantages such as the variation of lipid composition depending on the growth conditions (Manzanares et al. 2011). Nevertheless, Malfeito-Ferreira et al. (1989) reported the use of solid media concretely YPD Agar (Yeast Extract, Peptone, Glucose) to standardise the growth conditions and minimise any variation in their fatty acid composition. The determination of fatty acid profiles have allowed to separate wine yeast species according their spoilage potential.

On the other hand, the study of the variations in yeast lipidic composition depending on the oenological conditions has been widely studied. Among others it has been applied to studies of stress adaptation in wine. Beltran et al. (2008) used this technique for analysing fatty acid and sterol composition of yeast cells during alcoholic fermentation at low temperature in different liquid media (synthetic media and grape must). Furthermore, Borrull et al. (2016) showed that the wine yeasts changed their squalene, sterol and fatty acid composition during the acclimation to ethanol for producing sparkling wines.

Consequently, as it has been mentioned, changes in the cell lipid composition have a remarkable importance according to the growth condition to allow and ensure a good viability of cells. In this way, fatty acids (FA) and sterols are the main targets and the easiest to analyse in order to study the behaviour of yeast under different growing conditions.

1.3.2. Fatty acid profiles of lactic and acetic acid bacteria

The cellular fatty acids of oenological bacteria has been less studied that yeast. However, the first application of gas-liquid chromatography (GC) to analyse fatty acids was performed for the classification of bacteria (Abel et al. 1963).

On one hand, we find several studies in relation to the FA composition of LAB, most of them applied to identification. Rizzo et al. 1987 used the GC to identify species of *Lactobacillus* on the basis of their FA and neutral monosaccharides, demonstrating that they were able to identify 11 of 14 species of *Lactobacillus*. In addition, other the composition of FA was studied in other species of bacteria as *Pediococcus* (Uchida & Mogi 1972) and *Leuconostoc* (Schmitt et al., 1989; Tracy and Britz, 1989). Moreover, there are also other studies about the changes in the FA composition depending on the oenological conditions. Margalef-català et al. (2016) included in their study the effect of glutathione, added to wine against stress, in membrane FA of LAB.

On the other hand, AAB are obligate aerobic bacteria, widespread in fermented beverages and capable to oxidise ethanol as substrate in acid acetic (Guillamón & Mas 2009). Some authors had worked on AAB identification with GC, Yamada et al. (1981) studied the

discrimination of many species of the genera *Gluconacetobacter* and *Acetobacter*, according to their fatty acids.

1.4. Metabolomic approach

Since few years with the development of mass spectrometry (MS) techniques, researchers have started to find a relationship between the genome and phenotype of microorganisms or complex organisms (plant and human cells) (Fiehn et al., 2000; Schwab, 2003). Metabolome analysis permits the identification and quantification of extracellular and intracellular metabolites with molecular mass lower than 1000 Da (Villas Bôas et al., 2005).

In oenology, the determination of the exometabolome, by-products of the metabolic activities of microorganisms, has been explored by several authors during alcoholic fermentation (Skogerson et al., 2009; Pinu et al., 2014a) or balsamic vinegar elaboration (Pinu et al., 2016). In regard to yeast cells, the analysis of the overall metabolome was carried out from cells cultured in liquid medium. Some studies focused on the effect of growing conditions such as low temperature (López-Malo et al., 2013), during Sauvignon juice fermentation (Pinu et al., 2014b), others studies have been related with the supplementation of metabolites in the growth medium such as phenylethyl alcohol (Han et al., 2012).

2. Objectives

The main objective of the present work is to characterize the lipidomic and metabolomic profiles of oenological microorganisms, especially *S.cerevisiae,* growing on a solid culture medium.

The specific objectives are:

- Study of the impact of the number of colony-forming units on the production of the easiest extractable lipid and intracellular metabolites over time.
- Effect of culture conditions like temperature and oxygen was analysed.
- Analysis of cellular total fatty acid profiles on different species of yeasts, lactic acid bacteria and acetic bacteria growing on the same solid culture medium.

3. Material and Methods

3.1. Microorganism strains and culture media

Thirteen wine microorganism strains were used: 4 commercial wine yeasts, 5 LAB and 4 AAB (Table 1).

Table 1. Microorcanism strains used in this study (American Type Culture Collection [a], Spanish Type Culture Collection [b], Belgian co-ordinated collections of micro-organisms/Laboratory for Microbiology of Ghent[c])

	Name	Source	Cuture media
Yeast			
Saccharomyces cervisiae	Lalvin-QA23	Lallemand	YPDA, GMA
Lachancea thermotolerans	Laktia	Lallemand	
Torulaspora delbruekii	Biodiva	Lallemand	
Metschnikowia pulcherrima	Flavia	Lallemand	
Lactic acetic bacteria			
Oenococcus oeni	PSU-1	ATCC[a]	GMA
Lactobacillus brevis	4121 T	CETC[b]	
Lactobacillus buchneri	4674	CETC[b]	
Lactobacillus hilgardii	4786 T	CETC[b]	
Pediococcus pentosaceus	4695 T	CETC[b]	
Acetic acid bacteria			
Acetobacter aceti	1261	BCCM/LMG[c]	GMA
Gluconacetobacter hansenii	1527	BCCM/LMG[c]	
Acetobacter pasteurianus	1262	BCCM/LMG[c]	
Gluconacetobacter xylinus	1515	BCCM/LMG[c]	

3.2. Yeast culture preparation

Inocula were prepared by rehydrating active dry yeast (ADY) from commercial yeast strains for 30 min at 30 or 37ºC in water according to supplier's instructions. After rehydration strains were grown in YPD liquid at 28ºC, then yeast cells were spread on different media. Generally decimal dilutions were used for counting colonies on YPDA (20 g/L of glucose (Panreac, Barcelona, Spain), 20 g/L of peptone (Panreac), 10 g/L of yeast extract (Panreac) and 17 g/L of agar (Panreac)). Grape Must Agar (GMA) was also used in this study to cultivate all types of microorganisms (yeasts, LAB and AAB). Its composition is the following Commercial White Grape must (Spar), 4.5 g/L of yeast extract (Panreac), pH 4.5 (Lafon-Lafourcade and Joyeux, 1979). Plates were incubated under different conditions.

- To study the effect of time and number of yeast colony-forming units (CFUs), QA23 yeast strain was incubated during 2, 3 and 7 days at 28 ºC in aerobic conditions.
- The effect of anaerobiosis and temperature was analysed incubating QA23 at 28ºC in aerobiosis and at 28ºC in anaerobiosis (10% CO2 atmosphere), during 2 and 3 days, and at 16ºC during 6 and 8 days in aerobiosis.
- To observe the effect of YPDA and grape must agar media on lipid composition of yeast species, the different yeast species (Table 2) were incubated during 2 days at 28ºC in aerobiosis.

3.3. Estimation of total cell number of colonies

The estimation of total cell number of yeast colonies was measured by Optical Density (OD) at λ 600 nm. 1, 2, 3 or 4 colonies were picked from YPDA and immediately dissolved in 400 µL of sterile water. After homogenisation the Optical Density of the decimal cell solution was determined at λ 600 nm. From the same decimal dilution cells were counted with a Neubauer chamber under a microscope.

3.4. Lactic acid bacteria culture preparation

LAB strains were grown in modified MRS liquid (55 g/L MRS (Scharlau, Barcelona, Spain) supplemented with, 4 g/L of D,L-malic acid (SigmaAldrich,), 5 g/L of fructose (Panreac), pH 5) at 28ºC. After, decimal dilutions were plated on GMA. Plates were incubated at 28ºC in anaerobic atmosphere, with 10 % CO_2 during 4 and 8 days, except for *O. oeni,* which was incubated during 8 and 11 days.

3.5. Acetic acid bacteria culture preparation

The same procedure was followed for AAB strains. Inocula were grown in liquid GY (10 g/L of glucose and 10 g/L of yeast extract). Then decimal dilutions were spread on GMA and grown at 28ºC during 2 and 6 days.

3.6. Metabolomic and lipidomic analyses.

3.6.1.Extraction procedure of metabolites

The extraction procedure was the same for all types of microorganisms. Briefly from the procedure of López-Martínez et al. (2014), one or more colonies grown on the different media were introduced in an Eppendorf microtube containing *ca* 100 mg of 0.2-mm glass beads (BioSpec Products, USA), 10 µL of ribitol (internal standard (ISM) for metabolomic approach) at 1 mg/mL (Sigma, Barcelona, Spain), 10 µL of α-cholestane (internal standard (ISL) for lipidomic approach) at 1 mg/mL (Sigma) and 400 µL of methanol-water (1:1, v/v). After a strong agitation microtubes were placed in a thermal bath (Fisher Bioblock Scientific, Aubagne, Francia) at 90 ºC during 5 min. After cooling, 800 µL of chloroform were added to the samples which were agitated

on an Orbit M60 (LabNet Biotécnica, Madrid, Spain) at 120 rpm during 20 min. Samples were centrifuged at 10,000 rpm during 2 min to separate the two phases. The upper aqueous phase and the lower organic phase were separated in two new Eppendorf microtubes. After that samples were dried in SC110 speed vacuum system (Savant Instruments, USA) for 4 h.

The dried residue of aqueous phase was redissolved and derivatized for 30 min at 70 ºC in 40 µL of 20 mg/mL methoxyamine hydrochloride in pyridine (Sigma), followed by a 30 min treatment at 70ºC with 40 µL of *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA, Sigma). For the dried residue of the organic phase, samples were derivatized with 80 µL of MSTFA at 70ºC during 30 min.

3.6.2.Gas chromatography-mass spectrometry analysis.

Gas chromatography (GC) was performed using an Agilent Technologies Network GC system 6890N connected to an HP computer with the ChemStation software (Agilent Technologies). Compounds were detected using an inert mass selective detector (MSD, model 5975, Agilent Technologies).

Metabolomic analysis. 2 µL of the cell extract were injected at a split ratio of 20:1 into a DB-5HT column (30 m × 0.25 mm × 0.1 µm; Agilent Technologies) with an automatic injector (7683B, Agilent Technologies). Helium was used as carrier gas at a constant flow of 1.0 mL/min. The injector temperature was 200 ºC. The column oven temperature was initially held at 80 ºC for 4 min and then increased first to 200 ºC at a rate of 5 ºC/min and then to 300 ºC at a rate of 25ºC/min, where it was held for 7 min. The MSD transfer temperature was 300 ºC. The MSD quadrapole and source temperatures were maintained at 180 ºC and 280 ºC, respectively. The MSD data were acquired in electronic ionisation scan mode at 70 eV within the range of 35 – 650 amu after a solvent delay of 3 min. Postrun analysis was performed with the Agilent MSD Chemstation. The relative abundance of each identified compound was calculated according to the respective chromatographic peak heights corrected with respect to the IS (Ribitol) peak height. Metabolites were identified using an in-house MS and NIST 2005 libraries.

Lipidomic analysis. 3 µL of the lipidic extract were injected at a split ratio of 5:1 in the same device and column as described above. The chromatographic conditions were: injector temperature was 300 ºC. The column oven temperature was initially held at 90 ºC for 1 min and then increased first to 320 ºC at a rate of 15 ºC/min and then to 380 ºC at a rate of 4 ºC/min, where it was held for 1 min. The MSD transfer temperature was 300 ºC. The MSD quadrapole and source temperatures were maintained at 150 ºC and 280 ºC, respectively. The relative abundance of each identified compound was calculated according to the respective chromatographic peak heights corrected with respect to the IS (α-cholestane) peak height. Metabolites were identified using in-house MS and NIST 2005 libraries.

3.7. Statistical analysis.

Each combination of strains per condition was analysed by three independent biological samples. ANOVA (Tukey honestly significant difference (HSD) test), Principal Component Analysis (PCA) and Discriminant Analysis (DA) were performed with the XLSTAT software 2018.7 package with a statistical level of significance of $P < 0.05$. (Addinsoft, USA).

4. Results and Discussion

4.1. Effect of time and number of yeast colony-forming units on lipid composition of S. cerevisiae QA23

The intracellular lipid composition of *S. cerevisiae* QA23 was studied in regards to the number of yeasts CFUs, from 1 to 4 CFUs, and on the growing time, 2, 3 and 7 days.

The identified lipids by GC-MS were unsaturated fatty acids (UFAs), saturated fatty acids (SFAs), with different chain length, sterol and squalene (SD table S1). These results are in agreement with the typical lipid composition of *S. cerevisiae*, which is mainly UFAs, palmitoleic (C16:1) and oleic (C18:1) acids, followed by SFAs, palmitic (C16:0) and stearic (C18:0) acids (Klug 2014). The main sterol identified was ergosterol, the mayor one present on *S. cerevisiae*. which have an influence on its physical state by modifying fluidity and permeability (Sharma 2006). Curiously squalene was detected but neither other sterols nor phospholipids or sphingolipids, which mainly work as precursors and structural (Klug 2014).

As observed in Figure 1, the significance level of lipid content followed a similar pattern of evolution in all groups (Fig.1), regarding to the number of CFUs. The differences were not significant in any case, regardless the CFUs. Nevertheless, irrespective of the number of CFUs the yeast lipid content changed over time. There was an evolution in relation to 7 days but no between 2 and 3 days. we found an exception for the case of four CFUs, in which the detector response of cell lipid content was not lineal.

We could classify the lipids in three clusters considering their evolutive trend over time. It decreased in the case of SFA (Fig.1A) and squalene (Fig. 1C) and increased over time in relation to UFA (Fig.1B). In the other hand the percentage of ergosterol (Fig.1D) increased until the third day and then decreased but no significantly, so we could not see an evolution over time.

It is the first time to our knowledge that the evolution over time of lipidome in yeast grown on solid media has been investigated. However, many works have been done with respect to lipid evolution on different fermentation conditions, because of alterations of the membrane lipids due to stress (Bardi et al. 1999; Torija et al. 2003). For example, as the fermentation progresses,

under the anaerobic conditions associated, yeasts cannot synthesise sterols or long-chain unsaturated fatty acids (Aranda 2011).

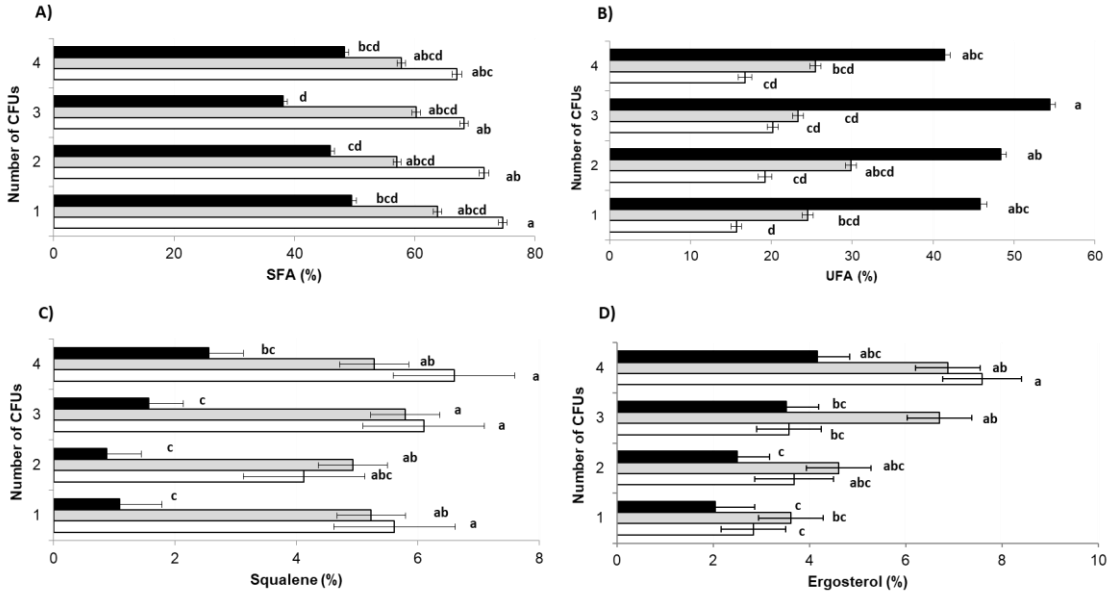

Figure 1. Effect of time (□ 2 days, ■ 3 day, ■ 7 day) on the lipid composition (%) of *S. cerevisiae* QA23 yeast cells according to the number of colony-forming units (CFUs). Saturated Fatty Acids (SFA) (A), Unsaturated Fatty Acids (UFA) (B), Squalene (C) and Ergosterol (D). Mean ± Standard Deviation (n = 3). Different lower-case letters indicate a significance difference among number of CFU using Tukey (HSD) Test at $P < 0.05$.

4.2. Effect of time and number of yeast colony-forming units on metabolome of S. cerevisiae QA23

From an average of more 150 peaks we found several metabolites (SD table S2). The most remarkable results are shown in Figure 2. They were clustered in four groups: phosphate, organics acids (succinic, fumaric, malic and citric acids), total amino acids (Total aa), which includes amino acids and some biogenic amines (putrescine and cadaverine) (SD table S3), and trehalose. In addition, others metabolites have been detected with certainty (>80% of quality) as lactic acid or glycerol.

It was observed that all metabolites followed an upward trend over time (Fig. 2). In almost all cases there were significant differences between 2 and 7 days, between 2 and 3 days, and between 3 and 7 days, with the exception of 1 CFU in all the clusters.

Regarding the number of CFUs, contrary to lipid composition, some significant changes were observed in the seventh day. No differences were found between number of CFUs on the second and third day. However, in PO_4^- (Fig.2A), organic acids (Fig. 2B) and trehalose (Fig. 2D) there were no differences for 3 and 4 CFUs in the seventh day. As well as in lipidome, the response of the detector was not lineal. The same happened between 2 and 3 CFUs in Total aa.

The evolution over time of the yeast metabolome on solid medium has not almost been subject of research, however in this investigation we found interesting behaviours. Interestingly enough is the increase of some amino acids such as ornithine and putrescine along the time. In yeast cells, ornithine is decarboxylated to putrescine which the main substrate for polyamine biosynthesis (Taborg et al., 1982). It should be noted that ornithine, putrescine and other polyamines not identified in our extract, but probably present, appear from the third day and increase in time. These metabolites are related with ageing cells and in response to Reactive Oxygen Species (ROS) generation (Eisenberg et al., 2009).

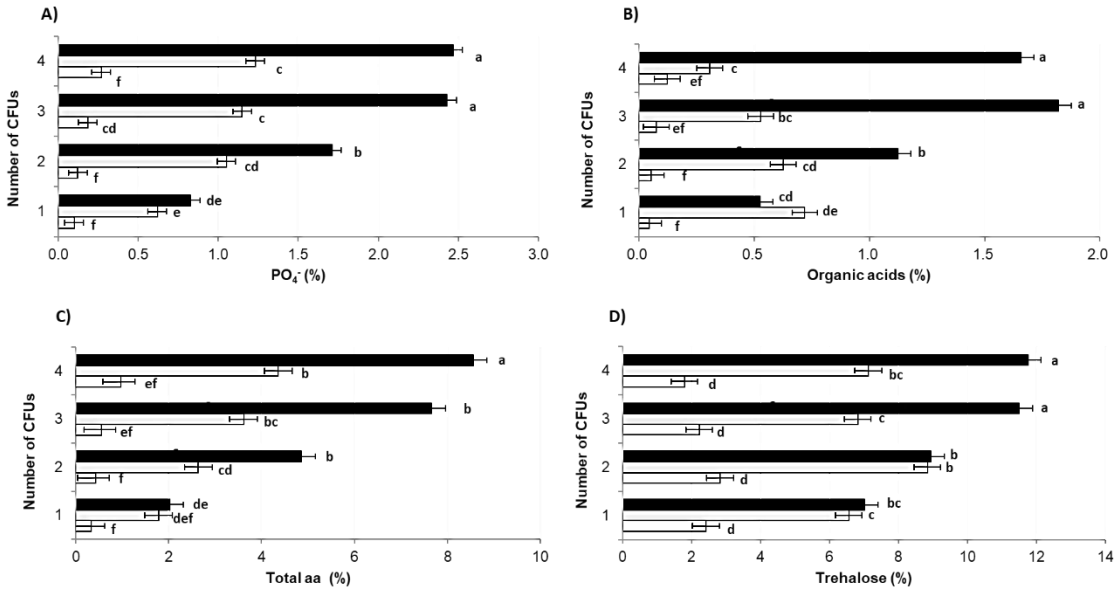

Figure 2. Effect of time (□ 2 days, ■ 3 day, ■ 7 day) on the metabolome (%) of QA23 yeast cells according to the number of colony-forming units (CFUs). (A), Organic acids (B), Total amino acids (Total aa) (C) and trehalose (D). Mean ± Standard Deviation (n = 3). Different lower-case letters indicate a significance difference among number of CFU and time using Tukey (HSD) Test at $P < 0.05$.

Metabolome results of yeast samples were subjected to a principal component analysis (PCA), shown in figure 3. The PCA was performed using the most significant metabolites: lactic acid, trehalose, total aa, organic acids and phosphate. It was obtained a model with two factors which explained the 97.45 % of the variance.

Samples were visibly clustered into three groups according to the day of growing: cluster 1: second day, cluster 2: third day and cluster 3: seventh day. It could be observed that, as we saw in the ANOVA results, in the third cluster there was a separation of the samples in relation to the number of CFUs. It was shown clearly that the variables responsible of this separation were total aa, PO_4^-, trehalose and organic acids. Also, it has to be pointed that there was a notorious separation of 1 CFUs samples.

In the second cluster there was a slight separation regarding to CFUs but no so differentiated, except with 1 CFUs samples. The separation was influenced by the variables lactic

acid and trehalose. Finally, in the first group there was not any separation of the samples, it was in the opposite direction of all the variables. It suggested that in the second day the number of CFUs had not influence on these variables.

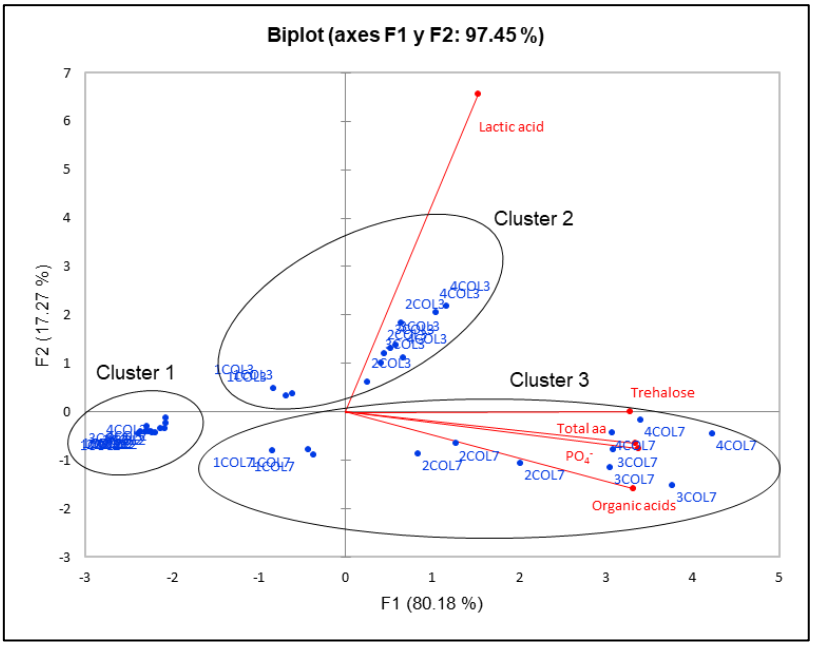

Figure 3. Biplot of Principal Component Analysis (PCA) using Lactic acid, trehalose, Total aa, PO_4^- and organic acids . Score plots of factor 1 (80.18%) against factor 2 (17.27%) where the samples were grouped on three clusters: Cluster 1: second day, Cluster 2: third day and cluster 3: seventh day.

In conclusion, regarding the number of CFUs there were not many differences neither in the lipid composition nor in the metabolome. It was enough one CFU to get a good signal. Because of that, the following experiments were carried out using only one CFU, which corresponds to 7.5 $\cdot 10^6$ cell/mL of *S. cerevisiae* (Table 2). So, avoiding the signal saturation observed with 4 and 3 CFUs in some cases.

Table 2. Relation between number of CFUs and number of cell/mL of S. cerevisiae QA23 counted under microscope. Mean ± Standard Deviation of three independent replicates.

Number of CFUs	cell/mL
1 CFU	$7.50 \cdot 10^6$ ±1.41E+05
2 CFUs	$1.06 \cdot 10^7$ ±8.49E+05
3 CFUs	$1.74 \cdot 10^7$ ±9.19E+05
4 CFUs	$2.23 \cdot 10^7$ ±1.41E+05

4.3. Effect of oxygen and anaerobiosis and temperature on lipid composition of S. cerevisiae QA23

To test the effects of absence of oxygen and low temperature, QA23 cells were plated after rehydration on YPDA and grown in aerobiosis and in a CO_2 atmosphere at 28ºC during 2 (Period 1) and 3 days (Period 2). Also, they were grown in aerobiosis at 16ºC but during 6 (Period 1) and 8 days (Period 2), due to the fact that the yeast grew slower at this lower temperature. The lipids identified were the same that in the experiment before (SD table S4).

Regarding to UFAs there were significant differences. The percentage of UFA decreased at low temperature, 16ºC (Fig. 4A). In contrast, UFA percentage did not change when the incubation time increased. These results are not in agreement with other studies. For instance, Torija et al. (2003) observed that UFAs increased with low temperatures. However, their study was carried out in concentrated must, not in solid media. What is more, the low temperature was even lower, 13ºC. In addition, it was detected that the unsaturation degree was different depending on the strain. In regards to MCFAs, it should increase their percentage in low temperature and in anaerobiosis (Beltran et al. 2008). However, in our study this MCFAs did not present significant differences in these conditions (SD table S4).

In relation to anaerobiosis some differences were observed in UFAs (Fig.4A) between 2 and 3 days, and a significant variation in the 2nd period with aerobiosis. The UFAs content in anaerobiosis at 28ºC showed an upward trend, while in the other conditions no significant changes were observed. Under anaerobiosis, yeasts should have lower levels of total lipids yeast growth normally requires added oxygen to synthesised lipids (sterols and UFAs) which are essential for plasma membrane integrity (Rattray et al. 1975). However, this increase trend could be explained as a response to stress (Steels et al. 1994).

On the contrary, the time evolution in relation to SFAs (Fig.4B) showed a descendent trend both under anaerobiosis and aerobiosis conditions at 28ºC; no differences over time were observed at 16ºC. Something similar happened for the ergosterol content (Fig.4C), significant differences were only found between the two periods of time in anaerobic condition. With respect to growing conditions do not appeared significant differences.

These results seem to contradict those of the literature, but for our point of view, they seem correct. Yeast cells grow more slowly on a solid medium than in a liquid medium especially at low temperatures. These lipid families should be analysed for 7-day culture cells.

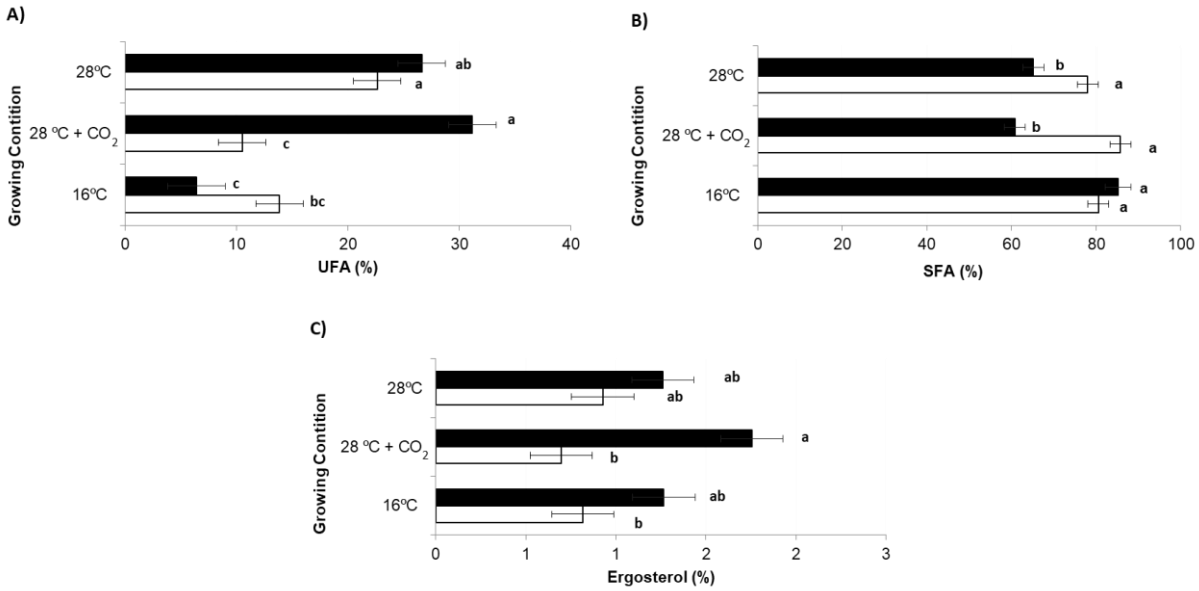

Figure 4. Effect of temperature and anaerobiosis on the lipid composition (%) of QA23 yeast cells on two different periods of time (□ 1st period, ■ 2nd period). Unsaturated Fatty Acids (SFA) (A), Saturated Fatty Acids (UFA) (B), Squalene (C) and Ergosterol (D). Mean ± Standard Deviation (n = 3). Different lower-case letters indicate a significance among growing conditions and time (HSD) Test at $p < 0.05$.

4.4. Differentiation of wine microorganisms by fatty acid profiling from the same growing solid medium

4.4.1.Effect of culture growth on lipid composition of yeast species.

Four different yeast species (*Lachancea thermotolerans, Metschnikowia pulcherrima, Torulaspora delbruekki and Saccharomyces cerevisiae*) were analysed to observe their lipidic composition in two different media, YPDA and GMA (SD table S5) .

There were not significant differences between the two media in the lipid composition of the tested yeasts. The percentage of SFA (Fig. 5A) did not change between YPDA and GMA. However, *T. delbruekii* presented significant differences on SFA in relation with the other species. Nevertheless, in the case of MCFA there were more variations between media but not statistically significant (Fig. 5B). Also, is noteworthy that *M. pulcherrima* did not present MCFA (Fig. 5B).

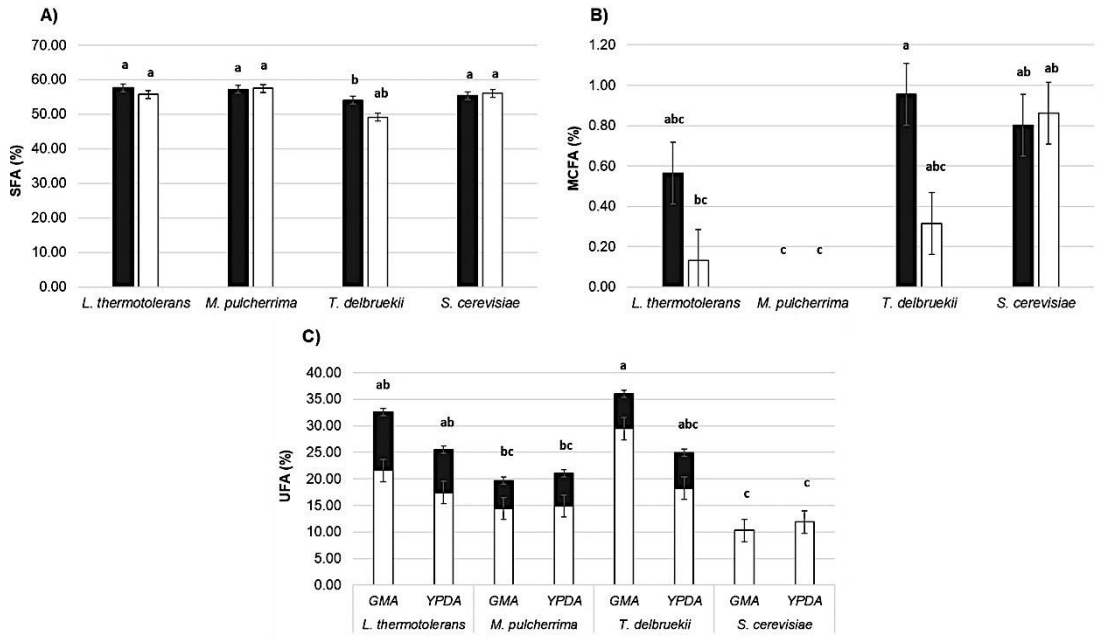

Figure 5. Effect of different media (A,B: □GMA ■YPDA) on the lipid composition (%) of different species: *L. thermotolerans, M. pulcherrima, T. delbruekki, S. cerevisiae*. Saturated Fatty Acids (A), Medium Chain Fatty Acids (MCFA) (B), Unsaturated Fatty Acids (UFA), separated in polyunsaturated fatty acids (PUFAs) and monounsaturated fatty acids (MUFAs) (C: □MUFAs, ■ PUFAs). Mean ± Standard Deviation (n = 3). Different lower-case letters indicate a significance among media and species (HSD) Test at $p < 0.05$.

Either there were not significant differences in UFA percentage between YPDA and GMA (Fig. 5C). However, there were remarkable differences on UFA composition between species (SD table S6). It is showed better in figure 6, in which only the variable species was taken into account and not the medium. Variations were found in both polyunsaturated fatty acids (PUFAs) and monounsaturated fatty acids (MUFAs) (Fig. 6). In particular we were able to differentiate *M. pulcherrima* and *S. cerevisiae*, due to the lack of PUFAs in *S. cerevisiae* and the production of linolenic acid (C18:3) by *M. pulcherrima*. Nevertheless, *L. thermotolerance* and *T. delbruekii* did not present significant differences on their UFA composition, which produce linoleic but no linolenic. In agreement with other studies, *S. cerevisiae* is characterized by the lack of ability to convert oleic acids into PUFAs, while some non-*Saccharomyces* species could produce PUFAs as linoleic (C18:2) and linolenic (C18:3) acids (Rozès,1992). For example, *T. delbruekii* known as *Saccharomyces delbrueckii* or *Saccharomyces rosei* or *Saccharomyces roseus*, can produce C18:3 depending on the *S. rosei* strain (Rozès, 1992), in our case the strain studied did not present this lipid.

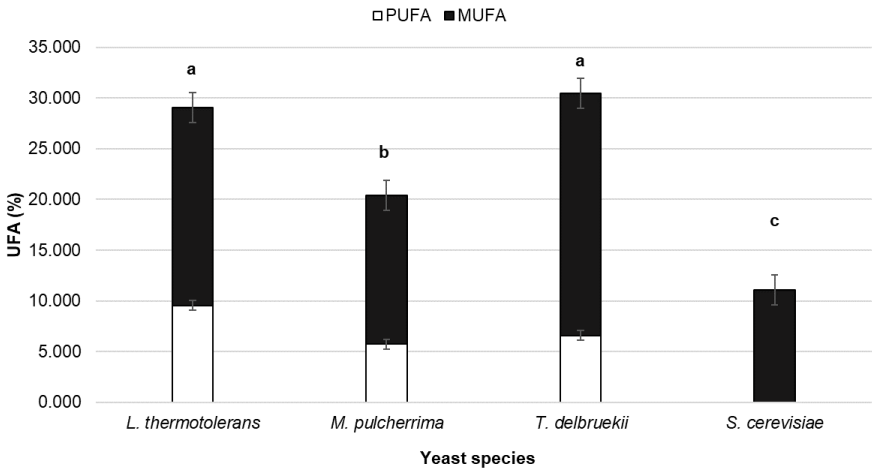

Figure 6. Percentages of Unsaturated Fatty Acids (UFA) separated into polyunsaturated fatty acids (PUFAs) and monounsaturated fatty acids (MUFAs), of four different yeast species: *L. thermotolerans, M. pulcherrima, T. delbruekki, S. cerevisiae*. Mean ± Standard Deviation (n = 3). Different lower-case letters indicate a significance among species (HSD) Test at $p < 0.05$.

4.4.2.Effect of GMA culture growth on lipid composition of LAB species

The variations of lipidome were also studied on five different LAB species: *Oenococcus oeni*, *Lactobacillus brevis*, *Lactobacillus hilgardii*, *Lactobacillus buchnieri* and *Pediococcus pentosaceus.* These LAB were grown in GMA media during 4 days, with the exception of *O. oeni* which was evaluated in the 11th day.

Regarding lipidome, the lipids detected were for SFAs, myristic (C14), palmitic (C16) and stearic (C18) acids, for UFAs, palmitoleic (C16:1) and oleic (C18:1) acids; and for Cyclopropane Fatty Acids (CFAs) (SD table S7). The last ones were detected as unknowns 1 and 2, but by bibliography, it is thought that might be lactobacillic and dihydrosterculic acids, respectively (Margalef-Català et al. 2016). There was a maximum percentage in *L. hilgardii* and a minimum in *O. oeni*, while that in the rest of species did not show significant differences (Fig. 7A).

The UFA (Fig.7B) allowed to differentiate two species, *P. pentosaceus,* with the highest percentage, and *L. buchnerii*. On the other hand, *L. brevis*, *L. hilgardii* and *O. oeni* did not present significant differences. In relation to SFA (Fig.7C), the unique species with significant differences was *O. oeni*, which present the higher value.

Rizzo et al. (1987) analysed the FA composition of different species of *Lactobacillus,* as *L. brevis* and *L. buchnieri*. In this study *L. buchneri* presented larger values of CFA and SFA and lower values of UFA than *L. brevis.* However, there were several differences between methods, for example in the extraction of metabolites. In the case of *P. pentosaceous* Uchida & Mogil (1972) studied the FA profile of species of *Pediococcus* showing an absence of lactobacillic acid and a presence of a correspondingly large amount of cis-vaccenic acid, which is the immediate precursor of lactobacillic acid. On the other part, there are only few studies of lipid composition of

O. oeni. Grandvalet et al. (2008) described a ratio of UFA to SFA close to 1 in *O. oeni* in the exponential phase grown under optimal conditions. This ratio decreased to around 0.6 in the stationary phase of growth. Our results showed a much higher percentage of SFA than of UFA. Since cells were grown 11 days, and this may correspond to an advanced stationary phase of growth, the low ratio UFA/SFA would be in accordance with the trend observed by Grandvalet et al. (2008). The higher ratio observed in this work could be due to strain variability and the differences in growth medium composition.

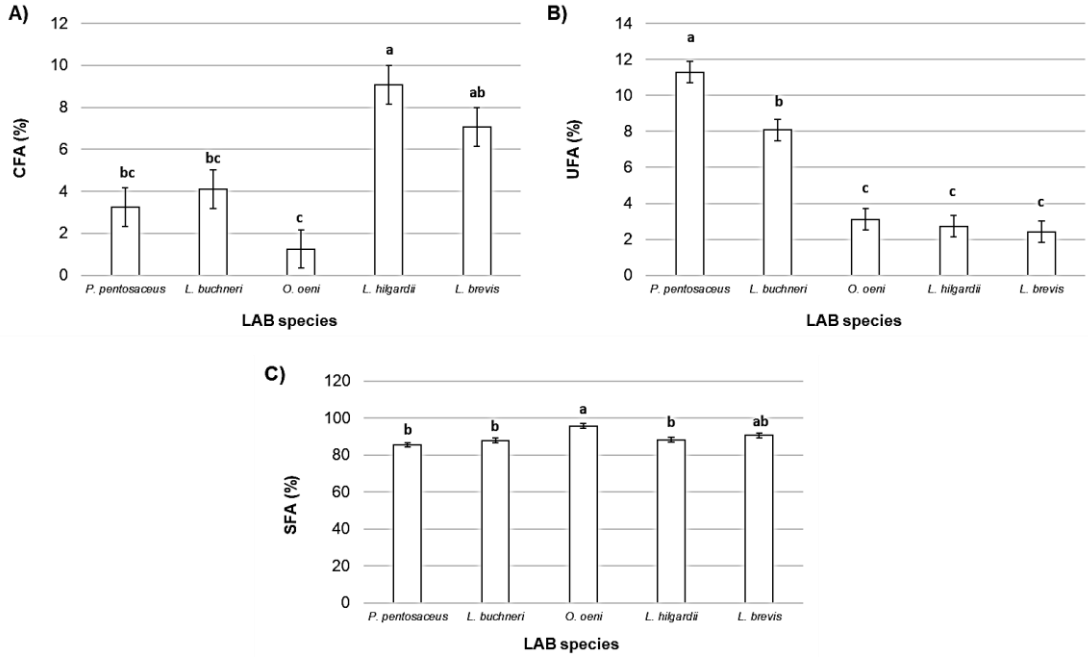

Figure 7. Percentages of Cyclopropane Fatty Acids (CFA) (A), Unsaturated Fatty Acids (UFA) (B) and Saturated Fatty Acids (SFA) (C) of five Lactic Acid Bacteria (LAB): *O. oeni, L. brevis, L. hilgardii, L. buchnieri and P. pentosaceus.* Mean ± Standard Deviation (n = 3). Different lower-case letters indicate a significance among species (HSD) Test at $p < 0.05$.

4.4.3.Effect of GMA culture growth on lipid composition of AAB species

In the case of AAB, we compared 4 species: *Acetobacter pasteurianus*, *Acetobacter aceti*, *Gluconacetobacter xylinum* and *Gluconacetobacter hanserii*. The fatty acids detected were SFA, myristic acid (C14), palmitic (C16) margaric (C17) and stearic (C18) acids; UFA just oleic acid (C18:1); and Hydroxy Fatty Acids (HFAs): 3-hydroxy myrisitc acid (3OH-C14), 2-hydroxy palmitic acid (2OH-C16), 3-hydroxy palmitic acid (3OH-C16), 3-hydroxy stearic acid (3OH-C18) and surely 12-hydroxy stearic acid (12OH-C18) (SD table S8). A 50% quality of the peak of last hydroxylated fatty acid matched with NIST data base.

HFAs were only found in AAB, in figure 8A is shown two groups significantly different, the genus *Acetobacter*, which had the highest percentages, and the genus *Gluconacetobacter.* Something similar happens with the HFA 3OH-C18 (Fig. 8B), but in this case we were able to

differentiate *A. pasteurianus* from *A. aceti.* No significant difference was found between the species of genus *Gluconacetobacter.*

Regarding SFAs (Fig.8C), there were not significant variations between *A. pasteurianus* and *A. aceti*. However, *G. xylinum* and *G. hansenii* were clustered in two different groups, presenting the higher and the lower values of SFA, respectively. Finally, in relation to UFAs (oleic acid) it was seen that *G. hanserii* had the largest values and A. aceti the lowest while A. pasteurianus and *G. xylinum* did not present significant differences (Fig.8D).

Yamada et al. (1981) studied the FA profile of different species of *Gluconacetobacter* and *Acetobacter*, including *A. aceti* and *A. pasteurianus* were among them. They study agrees with ours in the differentiation of *A. pasteurianus* and *A. aceti* according to their percentages of SFA. However, they discriminate the genera *Gluconacetobacter* and *Acetobacter* on the basis of the presence of the FA C14. Thus, it was not in agreement with our study in which the C14 was found. In addition, they could not find variations between the HFA and UFA of both genera.

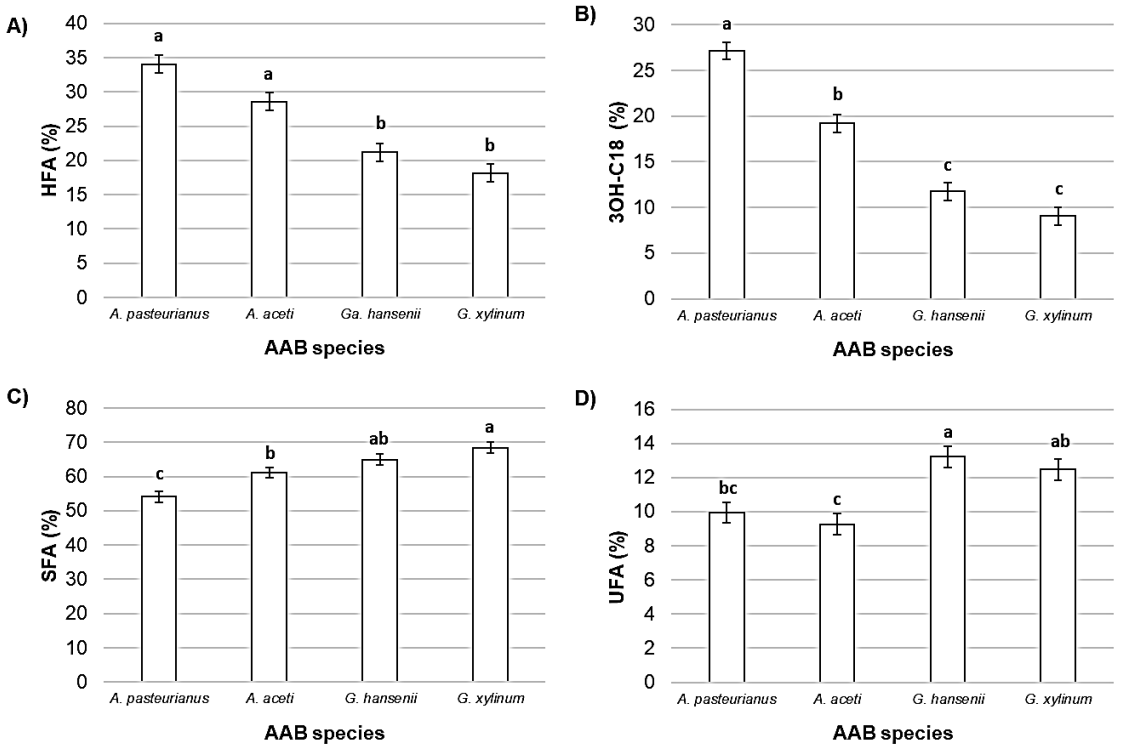

Figure 8. Percentages of Hydroxylated Fatty Acids (HFA), 3OH-C18, Saturated Fatty Acids (SFA) and Unsaturated Fatty Acids (UFA) of four Acetic Acid Bacteria (AAB): *A. pasteurianus*, *A. aceti*, *G. xylinum* and *G. hanserii.* Mean ± Standard Deviation (n = 3). Different lower-case letters indicate a significance among species (HSD) Test at $P < 0.05$.

4.5. Discriminant analysis of yeast, LAB and AAB

Many authors have used the total fatty acid (FA) profiling to identify microorganisms: yeast (Viljoen et al., 1988; Malfeito-Ferreira et al., 1989; Rozès et al., 1992; Botha et al., 1993), LAB (Uchida and Mogi, 1972; Rizzo et al., 1987; Schmitt et al., 1989; Tracy and Britz, 1989) and AAB, (Yamada et al., 1981; Franke et al., 1999). In contrasts, few of these have determined the FA composition from cells growing on solid media; YPDA for yeast (Malfeito-Ferreira et al. 1989) and different solid media for LAB and AAB (Li et al., 2014)).

In this study, it has been possible to provide a universal media to observe the behaviour of FA composition in the three types of microorganisms. This way we have been able to compare and create a model to classify the different species of wine microorganisms.

Two discriminant analysis (DA) were used to classify some "oenological" microorganisms in groups from a set of variables about the strains. The first one (DA1) was built from the results of fatty acid families (MCFA, SFA, MUFA, PUFA, CFA and HFA). The decided *a priori* clusters are the following: *S. cerevisiae* (only QA23 strain), non-*Saccharomyces* (*L. thermotolerans, M. pulcherrima and T. delbruekki*), LAB (*O. oeni*, *L. brevis*, *L. hilgardii*, *L. buchnieri* and *P. pentosaceus*) and AAB (*A. pasteurianus*, *A. aceti*, *G. xylinum* and *G. hanserii*). The second DA (DA2) was constructed by the following groups of species*: S. cerevisiae*, *L. thermotolerans*, *M. pulcherrima, T. delbruekki*, *O. oeni*, *L. brevis*, *L. hilgardii*, *L. buchnieri*, *P. pentosaceus, A. pasteurianus*, *A. aceti*, *G. xylinum* and *G. hanserii*. As the DA1, the same variables were used.

Firstly, it was obtained a model with two factors which explained 83.18% of the variance (Fig.9). The first factor (F1) separated yeast from bacteria, and the second factor separated *S. cerevisiae* from non-*Saccharomyces* and LAB from AAB. As it is shown in Figure 10 the CFA and the SFA were the main responsible variables of the LAB separation, while the HFAs was the most significant one in the separation of AAB. On the other hand, *S. cerevisiae* was more influenced by the variable SFA and MCFA, and non-*Saccharomyces* by the variables, PUFA and MUFA.

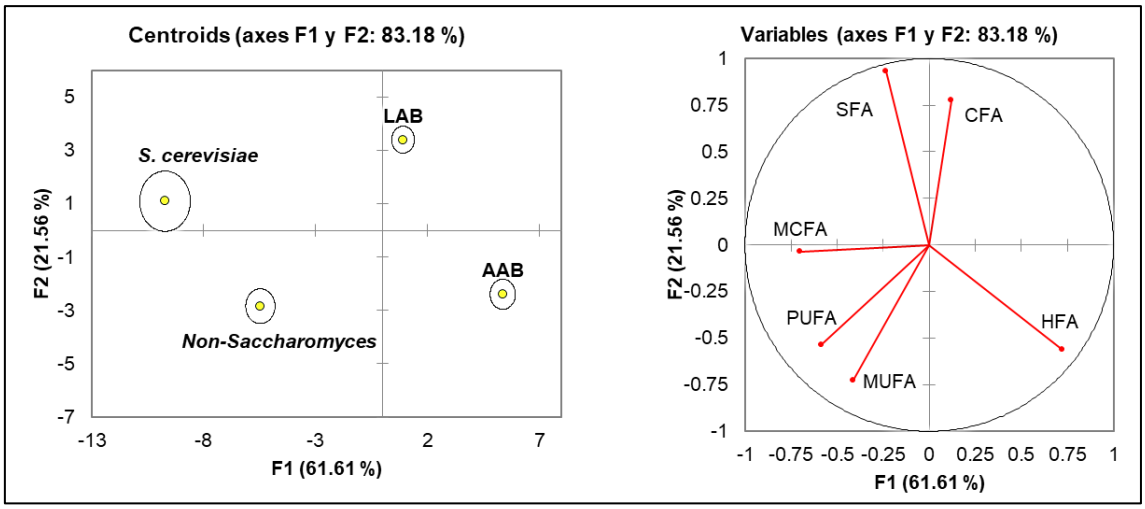

Figure 9. Discriminant analysis (DA1). Centroids of the four groups (A): *S.cerevisiae,* Non-*Saccharomyces*, Lactic Acid Bacteria (LAB) and Acetic Acid Bacteria (AAB*)*, and the following variables used (B): Saturated Fatty Acids (SFA), Medium Chain Fatty Acids (MCFA), Polyunsaturated Fatty Acids (PUFA), Monounsaturated Fatty Acids (MUFA), Cyclopropane Fatty Acids (CFA), and Hydroxylated Fatty Acids (HFA).

To evaluate the performance of the model to describe future samples, the samples were divided into two groups. The first one was defined with a triplicate of each group with the exception of *S. cerevisiae*, and the other with the remaining ones. Second group was used to construct the model, and the first group to evaluate if the model was able to classify correctly into the microorganism groups (Table 3) (SD Fig. S1).

Table 3. Validation matrix

Validation matrix						
de \ a	AAB	LAB	Non-*Saccharomyces*	*S.cerevisiae*	Total	% right
AAB	3	0	0	0	3	100
LAB	0	3	0	0	3	100
Non-*Saccharomyces*	0	0	3	0	3	100
S. cerevisiae	0	0	0	0	0	0
Total	3	3	3	0	9	100

As Table 3 shows, the model was able to perform 100% good classification in every microorganism group. It means this analytical methodology could be used as an identification method.

Secondly, another DA (Fig. 10) was made. In this case, the two factors explained the 86.04% of the variance. F1 discriminated AAB species from the rest of microorganisms, on the basis of HCFs variable. It was possible to observe the two genera of AAB clearly separated. There was more separation between the two species of *Acetobacter* genus, while *G. xylinus* and *G. hansenii* appeared closer. Regarding to F2, LAB and the yeast were separated. It could be observed that LAB were less separated. However, *L. hilgardii* and *L. brevis* were distanced from

L. buchnieri, O. oeni and *P. pentosaceus.* The key variables in this case were SFA and CFA. In regards to yeasts, the four species were clearly separated, mainly by MCFA, PUFA and MUFA.

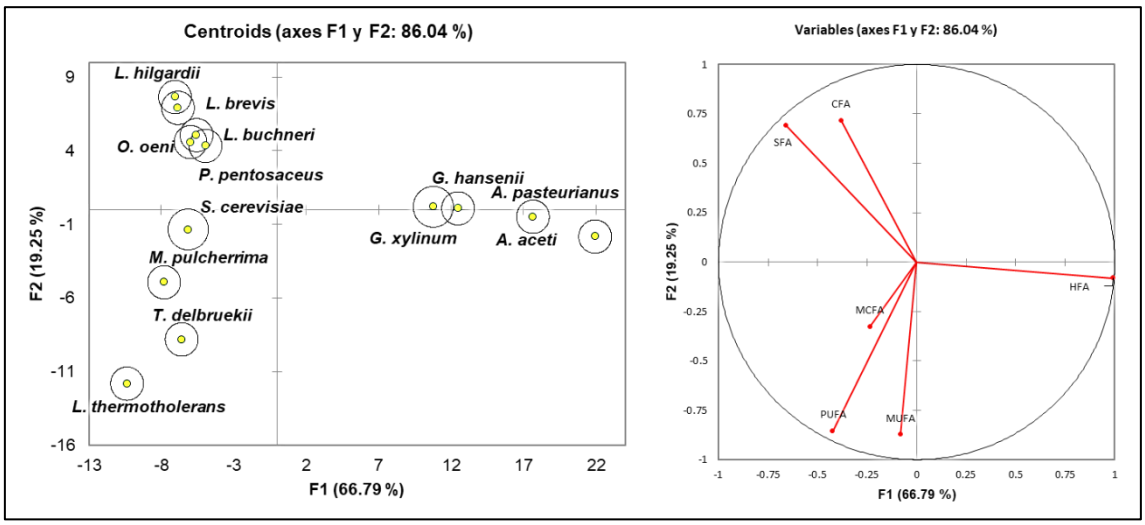

Figure 10. Discriminant analysis (DA). Centroids of the different species (A) and the following variables used (B): Saturated Fatty Acids (SFA), Medium Chain Fatty Acids (MCFA), Polyunsaturated Fatty Acids (PUFA), Monounsaturated Fatty Acids (MUFA), Cyclopropane Fatty Acids (CFA), and Hydroxylated Fatty Acids (HFA).

5. Conclusions and future perspectives

To sum up, we can conclude that we have been able to carry out a lipidome approach of microorganisms grown on a universal solid medium by using gas chromatography. Firstly, studying the lipidome profiles of *S. cerevisiae* grown on YPDA we have seen differences between several parameters and conditions: number of CFUs, time, temperature and anaerobiosis. In addition, a yeast metabolome approach was included. Secondly, we performed two discriminant analysis models based on the comparation of the total FA profiles of different yeasts, LAB and AAB species grown on the same universal media. We could highlight different conclusions:

- Significative changes have shown in lipidic composition of yeast over time, especially in the 7th day.
- The quantity of CFUs has not influence on the yeasts, LAB and AAB lipidome; one CFU is enough to determine the fatty acid profile of any "oenological" microorganisms plated on the same solid medium (GMA). It could be more advantageous and economical than the liquid condition due to the reduction in volume and the increase in the number of cells tested simultaneously on plates,
- It has been possible to make a metabolic and lipidomic screening regarding the significant differences founded between conditions, species and groups microorganisms.
- Two models have been done based on a DA, being able to classify oenological microorganisms on groups and species. It could be used like a fast technique for microorganisms identification.

In future investigations, the study of metabolome in all these microorganisms is going to be performed. This way, it will be established metabolomic and lipidomic profiles of oenological yeasts or bacterial cells, which could allow rapid screening of metabolic responses to different conditions, as activators or inhibitors. Moreover, the study should be completed with more species and even more strains to improve the model performance.

On the other hand, it should be notice that the method proposed could be used like a fast and reliable way to identify oenological microorganisms. In just a few hours it could be possible to have the lipidome and metabolome profiles to identify and compare it with the data base of the discriminant models. In addition, increasingly, there are big wineries which has a R&D department equipped with gas chromatographs; so, this way, apart from its use in research centres, it could be presented as a very interesting proposal for this type of enterprises.

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“Muere lentamente quien no arriesga lo cierto ni lo incierto por ir detrás de un sueño.
Vive hoy, arriesga hoy, hazlo hoy. “

-P. Neruda-

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9. ANNEX I. SUPPLEMENTARY DATA

Table S1. Summary of Least Square means (LS means) of QA23 lipidome (%). Different lower-case letters indicate a significance among number of CFUs and time using tukey (HSD) Test at $p < 0.05$.

	C10	C12	C14	C16:1	C16	C18:1	C18	Squalene	Ergosterol	MCFA	SFA	UFA
3 days *4 UFCs	2.538 a	2.108 a	2.502 a	8.143 a	39.925 a	17.289 a	15.339 bc	5.280 ab	6.875 ab	4.646 a	57.767 abcd	25.432 bcd
3 days *3 UFCs	2.098 ab	1.831 ab	2.513 a	7.767 a	40.776 a	15.558 a	16.966 abc	5.793 a	6.698 ab	3.930 ab	60.255 abcd	23.324 cd
3 days *1 UFC	1.727 abc	1.807 ab	2.602 a	12.183 a	38.650 a	17.688 a	15.810 abc	4.929 ab	4.604 abc	3.534 abc	57.062 abcd	29.871 abcd
3 days *2 UFCs	1.319 bcd	2.207 a	3.496 a	27.824 a	34.730 a	13.581 a	10.122 c	2.560 bc	4.161 abc	3.527 abc	48.348 bcd	41.405 abc
7 days*4 UFCs	1.299 bcd	1.492 abc	2.200 a	12.355 a	42.621 a	12.175 a	19.005 abc	5.233 ab	3.620 bc	2.792 bcd	63.826 abc	24.530 bcd
2 days*4 UFCs	0.920 cd	1.082 bc	1.696 a	6.985 a	43.747 a	9.773 a	21.611 abc	6.600 a	7.587 a	2.003 cd	67.053 abc	16.758 cd
2 days*3 UFCs	0.812 cd	1.495 abc	2.749 a	37.833 a	29.367 a	10.512 a	13.858 bc	0.872 c	2.501 c	2.307 bcd	45.974 cd	48.345 ab
7 days*1 UFC	0.794 d	1.150 bc	2.483 a	13.413 a	40.315 a	6.748 a	25.427 ab	6.098 a	3.571 bc	1.945 cd	68.225 ab	20.161 cd
2 days*1 UFC	0.866 cd	1.475 abc	2.295 a	37.493 a	28.086 a	16.947 a	7.750 c	1.567 c	3.521 bc	2.341 bcd	38.131 d	54.440 a
7 days*3 UFCs	0.386 d	1.030 bc	2.348 a	11.042 a	37.021 a	8.204 a	32.167 a	4.123 abc	3.679 abc	1.416 d	71.536 ab	19.246 cd
7 days*2 UFCs	0.487 d	0.722 c	2.350 a	8.458 a	44.182 a	7.226 a	28.121 ab	5.615 a	2.840 c	1.209 d	74.653 a	15.684 d
2 days*2 UFCs	0.517 d	0.995 bc	2.186 a	31.944 a	31.474 a	13.873 a	15.886 abc	1.086 c	2.040 c	1.512 d	49.545 bcd	45.817 abc

Table S2. Summary of LS means of QA23 metabolome (%). Different lower-case letters indicate a significance among number of CFUs and time using tukey (HSD) Test at $p < 0.05$

	PO_4^-	Orgnic acids	Total aa	Trehalose
3 days *4 UFCs	2.467 a	1.659 a	8.550 a	11.755 a
3 days *3 UFCs	2.430 a	1.820 a	7.656 a	11.508 a
3 days *1 UFC	1.712 b	1.124 b	4.861 b	8.941 b
3 days *2 UFCs	1.232 c	0.719 c	4.360 b	7.125 bc
7 days*4 UFCs	1.052 cd	0.528 cd	2.642 cd	8.840 b
2 days*4 UFCs	1.150 c	0.625 c	3.618 bc	6.821 c
2 days*3 UFCs	0.828 de	0.526 cd	2.025 de	7.022 bc
7 days*1 UFC	0.619 e	0.307 de	1.784 def	6.558 c
2 days*1 UFC	0.269 f	0.122 e	0.975 ef	1.792 d
7 days*3 UFCs	0.185 f	0.074 e	0.562 ef	2.226 d
7 days*2 UFCs	0.122 f	0.053 e	0.426 f	2.830 d
2 days*2 UFCs	0.100 f	0.043 e	0.330 f	2.418 d

Table S3. Total aa identified on QA23 metabolome.

Aliphatic aa	Aromatic aa	Hydroxylic aa	Sulphur- containing aa	Amidic aa	Acidic aa	Basic aa	Non-proteinogenic aa	Polyamines
Glycine	Phenylalanine	Serine	Cysteine	Glutamine	Glutamic acid	Lysine	γ-aminobutyric acid (GABA)	Putrescine
Alanine	Tryptophan	Threonine		Asparagine	Aspartic acid	Histidine	Citrulline	Cadaverine
Leucine	Tyrosine						Ornithine	
Valine								
Isoleucine								
Proline								

Table S4. Summary of LS means of QA23 lipidome (%). - Different lower-case letters indicate a significance among period and growing condition using tukey (HSD) Test at $p < 0.05$.

	C10	C12	C14	C16:1	C16	C18:1	C18	Squalene	Ergosterol	MCFA	SFA	UFA
2*16ºC	2.957 a	1.633 a	3.560 a	2.794 b	56.024 a	3.629 c	25.682 abc	1.508 a	1.267 ab	5.536 a	85.267 a	6.423 c
2*28 ºC + CO_2	1.444 ab	1.493 a	1.537 b	19.509 a	43.544 b	11.641 a	15.782 d	2.076 a	1.755 a	4.156 a	60.864 b	31.150 a
2*28ºC	1.348 ab	1.447 a	2.266 ab	16.962 a	43.443 b	9.675 ab	19.444 cd	2.811 a	1.261 ab	4.138 a	65.153 b	26.637 a
1*28ºC	0.721 b	1.138 ab	2.369 ab	15.041 a	54.283 a	7.585 bc	21.369 bcd	1.293 a	0.928 ab	4.264 a	78.021 a	22.626 ab
1*16ºC	0.440 b	0.481 b	1.862 b	7.501 b	52.332 a	6.344 bc	26.406 ab	1.996 a	0.817 b	2.742 a	80.601 a	13.845 bc
1*28 ºC + CO_2	0.630 b	0.827 ab	1.655 b	6.389 b	56.287 a	4.119 c	27.827 a	0.955 a	0.696 b	3.671 a	85.768 a	10.508 c

Table S5. Summary of LS means of fatty acids (%) of yeasts species. *Different lower-case letters indicate a significance among culture media and species using tukey (HSD) Test at p < 0.05.*

	C10	C12	C14	C16:1	C16	C18:3	C18:2	C18:1	C18	MCFA	SFA	UFA	MUFA	PUFA
***T. delbruekii]*YPDA**	0.586 a	0.368 ab	1.679 a	9.376 a	47.988 abc	0.000 b	6.633 bc	8.868 bc	23.811 bc	0.954 a	56.299 a	24.876 ab	18.244 bc	6.633 b
***L. thermotolerans*YPDA**	0.223 ab	0.340 ab	1.539 a	8.877 a	47.966 abc	0.000 b	8.108 ab	8.570 bc	23.172 c	0.564 abc	57.613 a	25.556 ab	17.448 bc	8.108 ab
***T. delbruekii*GMA**	0.315 ab	0.000 b	1.343 a	11.155 a	41.225 c	0.000 b	6.577 bc	18.327 a	19.814 c	0.315 abc	49.145 b	36.058 a	29.481 a	6.577 b
***L. thermotolerans*GMA**	0.000 b	0.130 ab	1.291 a	10.959 a	43.461 bc	0.000 b	10.981 a	10.630 b	21.714 c	0.130 bc	55.734 a	32.571 ab	21.589 ab	10.981 a
***S. cerevisiae*YPDA**	0.242 ab	0.559 a	1.325 a	5.530 a	54.006 a	0.000 b	0.000 d	6.350 bc	29.606 ab	0.801 ab	55.331 ab	11.880 c	11.880 bc	0.000 c
***S. cerevisiae*GMA**	0.226 ab	0.635 a	1.282 a	5.011 a	54.783 a	0.000 b	0.000 d	5.290 c	29.933 a	0.861 ab	56.065 a	10.300 c	10.300 c	0.000 c
***M. pulcherrima*GMA**	0.000 b	0.000 b	1.030 a	7.372 a	52.648 a	1.422 a	3.828 c	7.044 bc	24.959 abc	0.000 c	57.505 a	19.666 bc	14.415 bc	5.250 b
***M. pulcherrima*YPDA**	0.000 b	0.000 b	1.200 a	6.755 a	51.127 ab	1.312 a	4.909 bc	8.129 bc	25.761 abc	0.000 c	57.236 a	21.105 bc	14.884 bc	6.221 b

Table S6. Summary of LS means of fatty acids (%) of LAB species. Different lower-case letters indicate a significance among species using tukey (HSD) Test at $p < 0.05$.

	C10	C12	C14	C16:1	C16	C18:3	C18:2	C18:1	C18	MCFA	SFA	UFA	MUFA	PUFA
T.delbruekii	0.450 a	0.184 b	1.511 a	10.265 a	44.607 b	0.000 b	6.605 b	13.597 a	21.813 c	0.634 ab	52.722 b	30.467 a	23.863 a	6.605 b
L. thermotolerans	0.112 b	0.235 b	1.415 a	9.918 a	45.713 b	0.000 b	9.545 a	9.600 b	22.443 bc	0.347 bc	56.673 a	29.063 a	19.518 ab	9.545 a
S.cerevisiae	0.234 ab	0.597 a	1.304 a	5.270 b	54.394 a	0.000 b	0.000 d	5.820 c	29.770 a	0.831 a	55.698 ab	11.090 c	11.090 c	0.000 c
M.pulcherrima	0.000 b	0.000 b	1.115 a	7.063 ab	51.887 a	1.367 a	4.368 c	7.586 bc	25.360 b	0.000 c	57.371 a	20.385 b	14.650 bc	5.736 b

Table S7. Summary of LS means of fatty acids (%) of LAB species*. Different lower-case letters indicate a significance among number of species using tukey (HSD) Test at p < 0.05.*

	C14	C16:1	C16	VACC	C18:1	C18	UK1	UK2	SFA	UFA	CFA
L. hilgardii	2.367 ab	1.296 a	62.407 ab	0.560 b	0.873 b	23.418 b	2.062 b	7.017 a	88.191 b	2.729 c	9.079 a
P. pentosaceus	2.475 ab	0.657 b	57.638 ab	3.096 a	7.536 a	25.344 b	0.383 b	2.872 b	85.457 b	11.289 a	3.255 bc
L. brevis	2.856 a	0.496 b	57.096 b	1.079 b	0.843 b	30.563 a	6.461 a	0.605 b	90.515 ab	2.418 c	7.067 ab
L. buchneri	2.365 ab	0.680 b	59.564 ab	0.818 b	6.579 a	25.887 ab	0.314 b	3.792 ab	87.816 b	8.078 b	4.106 bc
O. oeni	1.975 b	0.641 b	63.133 a	1.804 ab	0.664 b	30.535 a	0.410 b	0.839 b	95.643 a	3.108 c	1.249 c

Table S8. Summary of LS means of fatty acids (%) of AAB species. Different lower-case letters indicate a significance among species using tukey (HSD) Test at $p < 0.05$.

	C14	3OHC14	C16	C17	2OHC16	C18:1	3OHC16	C18	3OHC18	12OHC18	SFA	HFA
G. xylinum	3.958 a	0.535 a	39.528 a	0.917 ab	3.822 a	12.481 ab	3.811 ab	24.941 a	9.044 c	0.963 a	68.427 a	18.175 b
A. pasteurianus	1.517 b	0.406 ab	29.721 b	1.945 a	0.511 b	9.943 bc	4.510 a	22.849 a	27.117 a	1.479 a	54.088 c	34.024 a
G. hansenii	2.022 b	0.000 c	38.577 a	0.601 b	5.693 a	13.213 a	2.831 ab	24.396 a	11.743 c	0.923 a	64.995 ab	21.190 b
A. aceti	2.126 b	0.301 b	37.543 a	0.966 ab	6.818 a	9.267 c	2.251 b	21.527 a	19.200 b	0.000 b	61.196 b	28.571 a

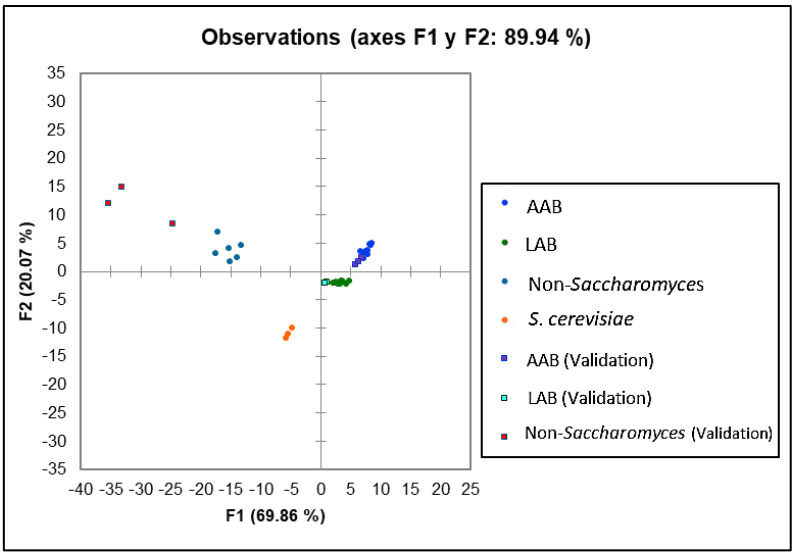

Figure S1. Discriminant analysis with validation. Samples of the four groups (A): ●*S. cerevisiae,* ● Non-*Saccharomyces*,● Lactic Acid Bacteria (LAB) and ● Acetic Acid Bacteria (AAB) and samples of validation: ■ AAB (Validation), ■ LAB (Validation), ■ Non- *Saccharomyces* (Validation