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Differentiation of Saccharomyces species by lipid and metabolome profiles from a single colony

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Abstract:	Yeast metabolism depends on growing conditions, which include the chemical composition of the medium, temperature, and growth time. In general, fatty acid profiles have been used to differentiate yeasts growing in liquid media. In this work, the fatty acids of Saccharomyces species were determined using colonies. In addition, using the same method, the effect of the number of colonies and growth time had on solid media allowed us to determine the metabolomic profiles of the cells. Our results showed that growth time modified the lipid and metabolomic profiles of the cells more than the number of colonies analyzed. For the first time, Saccharomyces cerevisiae and S. bayanus were differentiated using the fatty acid profile of one colony. Furthermore, other Saccharomyces species were separated from the two previously mentioned species. The synthesis of saturated fatty acids was greater than that of unsaturated fatty acids during the first two days of cell growth on a solid medium compared to a liquid medium. Unsaturated fatty acids subsequently became predominant.
Response to Reviewers:	Detailed responses to reviewer 1 Manuscript Number: FM-D-21-00411R2, Fourth revision Modified title: Differentiation of Saccharomyces species by lipid and metabolome profiles from a single colony Authors: Candela Ruiz-de-Villa et al. First, we again thank the reviewer for his efforts to help us improve the article again. The authors edited the manuscript based on the reviewer's suggestions, which are marked in red in the new version. Reviewer #1: The authors have addressed all my previous comments satisfactorily. Just a few minor language issues are highlighted below. Line 18: "the effect that" instead of "the effect of" Line 22: "were" instead of "have been" Line 23: "indeed" instead of "concretely" Line 123: "all the above-mentioned studies were" Line 124: "to our knowledge, the production over time of intracellular metabolites during yeast colony growth has never been investigated." Line 444: "than in" should not be in itàlics Line 480: the hyphen should be replaced by a comma; "two of these strains" instead of "these strains" We have followed the reviewer's suggestions and the changes appear in red in the text.

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Editor in Chief of Food Microbiology

Tarragona, 3rd December 2021

Dear Dra. Aline Lonvaud-Funel,

Again, I am sumitting a new revised version of the article "**Differentiation of *Saccharomyces* species by lipid and metabolome profiles from a single colony**", authored by Candela Ruiz-de-Villa, Montse Poblet, Albert Bordons, Cristina Reguant and Nicolas Rozès.
The manuscript has been revised based on the reviewer's latest suggestions. Responses and changes recommended by the reviewer are marked in red in the new manuscript.

We hope that this review will now be deemed acceptable for publication in the journal Food Microbiology.

Yours sincerely,

Nicolas Rozès

Detailed responses to reviewer 1

Manuscript Number: FM-D-21-00411R2, Fourth revision

Modified title: **Differentiation of *Saccharomyces* species by lipid and metabolome profiles from a single colony**

Authors: Candela Ruiz-de-Villa et al.

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Highlights

- Lipid and metabolome profiles of *Saccharomyces* species growing on YPD agar were determined through GC-MS.
- We differentiated the *Saccharomyces* species from the fatty acid profile using one colony.
- Higher ratio of SaturatedFA/UnsaturatedFA in yeast cells growing on a solid than in liquid medium after 2 days of growth.

Differentiation of *Saccharomyces* species by lipid and metabolome profiles from a single colony

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Abstract

Yeast metabolism depends on growing conditions, which include the chemical composition of the medium, temperature and growth time. Historically, fatty acid profiles have been used to differentiate yeasts growing in liquid media. The present study determined the fatty acids of *Saccharomyces* species in colonies. Using the same method, the effect ~~of~~ that the number of colonies and growth time had on solid media allowed us to determine the metabolomic profiles of the cells. Our results showed that the lipid and metabolomic profiles of the cells evolved as the colony grew. Interestingly, some strains of *Saccharomyces cerevisiae* ~~have been~~ were differentiated using the fatty acid profile of a colony; ~~concretely~~ indeed EC1118 and QA23 strains were separated from ICV-K1 and BM4x4. The synthesis of saturated fatty acids was greater than that of unsaturated fatty acids during the first two days of cell growth on a solid medium compared to a liquid medium. Unsaturated fatty acids subsequently became predominant. Finally, this methodology could be useful for carrying out physiological studies in a complete or defined solid growth medium allowing the supplementation of compounds, which inhibit or activate the growth of yeasts.

Keywords

Wine yeast; fatty acids; trehalose; squalene; *Saccharomyces cerevisiae; S. uvarum*; *S. kudriavzevii*

1. Introduction

Microbial diversity, both in vineyards and cellars, plays an important role in winemaking. Yeasts and lactic acid bacteria (LAB) are crucial to winemaking, as they are responsible for alcoholic and malolactic fermentations, respectively (Fugelsang & Edwards, 2007). The dominant species in alcoholic fermentation are *Saccharomyces cerevisiae* and the closely related *Saccharomyces bayanus*, due to their efficient fermentative catabolism (Pretorius, 2000). A wide variety of commercial *S. cerevisiae* strains –including *S. bayanus* ones, sometimes mistakenly labelled as *S. cerevisiae*– are used as starter cultures. In the wine industry, it is argued that the use of these starters is associated with a loss of wine typicity (Philip et al., 2021) but there is no clear scientific evidence for its existence. For this reason, it is increasingly common for wineries to isolate and select indigenous *S. cerevisiae* strains that are associated with particular characteristics typical of wines of the area (Tempère et al., 2018). In addition to *S. cerevisiae* and *S. bayanus*, there are other interesting species in the *Saccharomyces* genus, such as *S. uvarum* and *S. kudriavzevii*. These species have been studied for their remarkable properties (Minebois, 2020), including their high production of volatile compounds (Gamero et al., 2013; Masneuf-Pomarède et al., 2010).

Nevertheless, under uncontrolled conditions, other undesirable yeast species may also be found (Pretorius, 2000). For this reason, microbiological control is necessary for avoiding spoilage, for analyzing intraspecific variability and for verifying the fermentation strains. Typically, to do this, the microorganisms involved in the process must first be isolated and then identified. Depending on the physiology of the microorganism, specific culture media are used for this isolation. For yeasts, the most widely used media are nutrient rich, the most common of which is yeast extract peptone dextrose agar (YPDA). Selective and

differential media, such as a medium with ethanol and sodium metabisulfite, can also inhibit the growth of sensitive microorganisms (Kish et al., 1983).

Several methods have been used to characterize and identify yeasts, from biochemical phenotypic tests to molecular techniques (Pincus et al., 2007). Examples include phenotypic yeast identification methods such as the rapid screening tests, the analytical profile index (API) system and the use of the previously described selective media. Nevertheless, in recent years these methods have been almost entirely supplanted by more accurate molecular biology techniques. Regardless of their discriminating power (species or strains), examples include DNA–DNA hybridization; karyotyping methods; fingerprinting methods such as interdelta polymorphism fingerprinting; and microsatellites (Querol, 1992; Ivey & Phister 2011).

Molecular techniques also include several culture-independent techniques, such as quantitative real-time polymerase chain reaction (q-PCR), and next-generation sequencing (NGS) techniques, which allow genetic polymorphisms to be detected through massive sequencing (Bokulich & Mills, 2012).

However, as suggested by Pincus et al. (2007), rapid conventional identification methods such as chromogenic media and rapid enzymatic methods allow rapid and presumptive detection of the most critical and common opportunists in the health sector. Another interesting example is obtaining the fingerprints of long-chain fatty acids of yeast by gas chromatography (GC) (Pretorius et al., 1999). This technique has been used in some studies since Abel et al. (1963) demonstrated that microorganisms can be classified into genera and species according to their lipid composition, which is determined using GC with a flame ionization detector (FID). Other studies include a rapid method of identifying several species of *Candida* based on the presence or absence of certain fatty acids in their lipid composition (Gunasekaran & Hughes, 1980), the differentiation of wine yeasts —*S.*

cerevisiae from other spoilage yeasts— by their composition in total fatty acids (Rozès et al., 1992), and the identification of different spoilage yeasts in a wine bottling plant (Malfeito-Ferreira et al., 1989). The latter study also reported the use of solid media YPDA to standardize growth conditions and minimize any variation in fatty acid composition.

On the other hand, other gas chromatography techniques have also been applied to metabolome analyses for identifying and quantifying extracellular and intracellular metabolites with molecular masses lower than 1000 Da (Villas Bôas et al., 2005). Several authors have focused on determining the exometabolome —i.e. by-products of yeast metabolic activities— during alcoholic fermentation (Skogerson et al., 2009; Pinu et al., 2014a; Minebois et al., 2020). Others have evaluated the effect of growth conditions, such as low temperature. (López-Malo et al., 2013) or during Sauvignon must fermentation (Pinu et al., 2014b). All the above-mentioned studies were ~~All this previous work was~~ carried out from liquid culture and to our knowledge, the production over time of intracellular metabolites during yeast colony growth has never been investigated. ~~no bibliographic results mention the study of intracellular metabolites of yeast during their growth in solid medium.~~

The aim of this study was to develop a new gas chromatography-mass spectrometry (GC-MS) method based on the lipid and metabolome profiles of a single picked colony to differentiate species of *Saccharomyces*. The optimization took into account culture media, number of colonies used, and growth time. In addition, this methodology could allow physiological studies to be performed in a complete or defined solid growth medium to assess the yeast response to inhibitor or activator compounds.

2. Materials and methods

2.1. Microorganism strains and culture media

Six *Saccharomyces* strains were used in this study (Table 1). The strains Lalvin QA23® and Lalvin EC 1118® are labelled as *Saccharomyces cerevisiae bayanus* by the manufacturer. Since this denomination is not clear regarding the species, we have chosen to name them as *S. cerevisiae*, following López-Malo et al. (2013).

The inocula were prepared from commercial active dry yeast (ADY) and from liquid frozen cells. The ADY was rehydrated according to the supplier's instructions: 30 min in water at 37 ºC. For cells from liquid frozen cultures~~,~~ the strains were first incubated in YPD liquid (20 g/L of dextrose, 20 g/L of peptone, 10 g/L of yeast extract (Cultimed, Barcelona, Spain)) at 28 ºC for two days. Then for all yeasts an overnight preculture in YPD was performed. Next, the yeast cells were spread onto solid media using decimal dilutions for colony counting. Two different media were used, the first was YPDA (YPD with 17 g/L of agar [Cultimed] at pH 4.5), and the second was liquid YPD. YPDA was used throughout the study for all the species and liquid YPD (20 mL) was only used to determine the fatty acid composition of the yeast cells grown statically overnight at 28 °C in an Erlenmeyer flask. The cells were harvested after centrifuging and the fatty acids were extracted and silylated, as explained below in the metabolite-extraction procedure.

To study the effect of time and determine the minimal number of yeast colonies, the strain *Lalvin* QA23 was used as a reference. It was incubated for two, three, and seven days at 28 ºC in aerobic conditions on YPDA. In contrast, the different *Saccharomyces* strains were incubated for two days at 28 ºC on YPDA (Table 1). One colony (diameter of ca 3 mm) was taken from the corresponding growth media to compare the lipid and metabolome profiles between the strains.

2.2. Estimation of total cell number in the colonies

The total cell number in a colony was estimated using the relationship between the optical density (OD) and the total cell number. The reference strain was also Lalvin QA23 and it was grown on YPDA at 28 ºC. Between one and four colonies were randomly picked and immediately resuspended in 400 µl of sterile water. After homogenization, the OD of the decimal cell solution was determined at λ 600 nm. From the same decimal dilution, the cells were counted using a Neubauer chamber under a light microscope (Leica Microsystems, Wetzlar, Germany).

2.3. Metabolite extraction procedure

The metabolome and lipid extraction procedure was the same for all the strains. Briefly, using a modified version of the procedure described in López-Martínez et al. (2014), the specified numbers of colonies grown on the different media were introduced into Eppendorf tubes containing ca 100 mg of 0.5-mm glass beads (BioSpec Products, Qiagen) and 400 µL of methanol-water (1:1, v/v). As internal standards (IS), 10 µL of ribitol at 1 mg/mL (Sigma-Aldrich, Barcelona, Spain) and 10 µL of α-cholestane at 1 mg/mL (Sigma-Aldrich) were used for the metabolomic and lipidomic approaches, respectively. After shaking vigorously with a vortex mixer (30 sec), the microtube was heated to 90 ºC for 5 min. Once cooled, 800 µL of chloroform was added and the tube was then shaken at 120 rpm for 20 min and centrifuged at 10,000 rpm for 2 min. The two phases were physically separated from one another. Finally, the aqueous and organic phases were dried in a SC110 speed vacuum system (Savant Instruments, USA) for 4 h.

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

phase (lipid), the samples were only derivatized with 80 μL of MSTFA at 70 ºC for 30 min.

2.4. Gas chromatography-mass spectrometry analysis

Both the metabolome and lipid profiles were analyzed using GC-MS, with a 6890N GC system (Agilent Technologies, Germany) equipped with a DB-5HT column (30 m × 0.25 mm × 0.1 μm; Agilent Technologies) and an automatic injector (7683B, Agilent Technologies). Helium was used as the carrier gas at a constant flow of 1.0 mL/min. The compounds were detected with a mass selective detector (MSD, model 5975, Agilent Technologies). The MSD temperatures were 300 ºC, 180 ºC and 280 ºC for the transfer, quadrupole, and source, respectively. The MSD data were acquired in electronic ionization scan mode at 70 eV within the 35–650 amu range after a solvent delay of 3 min and then analyzed. using the Agilent MSD Chemstation software (Agilent Technologies). The metabolites were identified using an in-house MS and the NIST 2005 libraries. The relative abundance of each identified compound was calculated according to the respective chromatographic peak heights corrected in relation to the IS peak height, ribitol and α-cholestane for metabolome and lipid analysis, respectively. The results were expressed as arbitrary units (AU).

2.5. Metabolomic analysis

Two μL of the derivatized cell extract were injected at a split ratio of 20:1 at an injector temperature of 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 25 ºC/min, where it was held for 7 min.

2.6. Lipidomic analysis

Three μL of the derivatized lipid extract were injected at a split ratio of 5:1 at an injector temperature of 300ºC. 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.

2.7. Statistical analysis

Each combination of strains per condition (number of colonies, growth time, strains and solid growth media) was analyzed using three independent biological samples. An ANOVA (Tukey honestly significant difference [HSD] test) and principal component analysis (PCA) were performed using the XLSTAT software 2018.7 package (Addinsoft, Paris, France) with a statistical significance level of $p < 0.05$.

3. Results and discussion

The composition of yeast cells, i.e., lipids (fatty acids, squalene and ergosterol), amino acids, sugars, organic acids and other metabolites, depends on the growing conditions, which include, among other factors, the chemical composition of the medium, and whether the conditions are aerobic or anaerobic (Klug, 2014; Malfeito-Ferreira et al., 1989; Manzanares et al., 2011). Therefore, the first objective of this work was to study the response of the *Saccharomyces cerevisiae* Lalvin QA23 strain over time, during its growth on YPD agar medium. In addition, the optimal number of colonies was verified in order to standardize the parameters of the method and to determine the minimum yeast population necessary to yield a reproducible detection in the GC analysis. Thus, one to four colonies were randomly picked from the same plate after two days and pooled together. The average number of cells according to each colony pool (1 to 4) was significantly different, ranging from $0.75 \cdot 10^7$ to $2.23 \cdot 10^7$ cells/mL (Table 2). However,

the relationship between the number of cells/mL and the colonies was well correlated (R^2 = 0.9822), which allows us to validate the results between the number of colonies and the metabolite profiles. Growth was analyzed at two, three and seven days to determine the best and minimum time to obtain well-differentiated metabolome and lipid profiles.

3.1. Effect of number of colonies and growth time on the lipid composition of strain *Lalvin QA23*

The lipids determined were grouped as unsaturated fatty acids (UFAs), saturated fatty acids (SFAs), sterol, and squalene (Figure 1). Palmitoleic (C16:1) and oleic (C18:1) acids were the main UFAs detected. Myristic (C14:0), palmitic (C16:0) and stearic (C18:0) acids were the SFAs identified. The lipid profiles of Lalvin QA23 identified by GC-MS agreed with the fatty acid composition described for *S. cerevisiae* (Klug, 2014); in other words no polyunsaturated fatty acids were detected. Under the growth conditions used (YPDA), the only medium-chain fatty acids (MCFAs, C6 to C12) detected were capric acid (C10) and lauric acid (C12), regardless of the growth time (Supplementary File ST1). These MCFAs are generally found in higher concentrations in hypoxic conditions, such as white winemaking or during the anaerobic growth of *Saccharomyces* and allow the cell to modulate its structural and functional membrane integrity (Beltran et al., 2008). The total ~~*S. cerevisiae*~~ fatty acids detected in strain Lalvin QA23 were distributed as follows: 50–55% oleic acid, 25–30% palmitoleic acid, 25–30% palmitic acid, 8–9% stearic acid, and only 1.8–3.4% MCFAs.

Interestingly, using our simple extraction procedure (colony resuspended in a two-phase solvent system and heating the cells for 5 min) we detected squalene, the precursor of sterol biosynthesis, and ergosterol, the final product of this biosynthesis in yeast. The detection of ergosterol, the main free sterol present in the membranes of yeast cells (Tuller

et al., 1999) was expected. Surprisingly, squalene, which is normally present in low percentage in yeast cells grown under aerobic conditions, has also been detected; no other sterol precursors were detected under our experimental conditions (growth culture and lipid determination).

No significant changes were found in percentages of SFA, UFA, squalene, and ergosterol according to the number of colonies (Figure 1). The major lipids identified were fatty acids, which represented around 87% of total lipids detected, regardless of how many colonies were analyzed (Figure 1A & 1B). In comparison, squalene and ergosterol were detected in the 3.3–4.8% and 2.8–6.2% ranges, respectively (Figure 1C and 1D). Nevertheless, the ergosterol percentage increased significantly from 1 to 4 colonies (Figure 1D), while the percentage of squalene remained constant (Figure 1C). Based on these results, analyzing just one colony should be sufficient to provide a statistically consistent lipid profile.

The evolution of yeast lipids as a function of growth time on solid media has not previously been described. However, many studies have evaluated lipid evolution under different fermentation conditions, due to the alteration of the membrane lipids in stressful environments (Bardi et al., 1999; Torija et al., 2003). For example, as fermentation progresses, under anaerobic conditions, yeasts cannot synthesize sterols or long-chain unsaturated fatty acids (Aranda, 2011). The only study that has analyzed yeast cell lipids from a colony was performed by Malfeito et al. (1997), using a large 2-cm diameter colony. In our study, we observed that increasing the growing time of colonies on YPDA played an important role in the ratios of total cell lipids (Figure 1). The same pattern was generally observed for all lipids, regardless of the number of colonies. On other hand, the percentage of SFA decreased over time as the number of colonies grew (Figure 1A), while the opposite pattern was observed for the percentage of UFAs: the longer the growth time,

the higher the percentage of UFAs (Figure 1B). With respect to the proportion of squalene found in the cells over time, the same pattern was observed as for SFA, in other words, percentages decreased as a function of growth time (Figure 1C). In addition, the percentage of ergosterol increased until the third day in all samples analyzed. However, the observed diminution at day 3 for four picked colonies could be due to the saturation of signal detection in our analytical conditions (Figure 1D).

3.2. Effect of number of colonies and growth time on the metabolome composition of strain *Lalvin QA23*

From an average of more than 150 peaks detected in one run, several substances ~~have been~~ were identified in the metabolic profiles of strain Lalvin QA23. Among these metabolites we identified amino acids, polyamines, organic acids, sugars (mono- and disaccharides), and polyols (sugar alcohols such as inositol, erythritol, 2,3-butanediol, glycerol, etc.). The main results are shown in Figure 2. The compounds were clustered in four groups: phosphate anions (PO_4^-); organic acids (succinic, fumaric, malic and citric acids); total amino acids identified (Total AA), which also included some biogenic amines (putrescine and cadaverine), and trehalose (Additional data displayed in Supplementary File ST1).

To the best of our knowledge, the temporal evolution of the yeast metabolome on plates of solid growth medium has never been studied, and that was one of the objectives of this work. Indeed, just as with lipidomic studies, all metabolomic analyzes using GC-MS are currently performed with extra- and intracellular yeast samples growing in different liquid media. Examples of this include the alcoholic fermentation of a defined medium (Minebois et al., 2020), the fermentation of grape juice (Ritcher et al., 2015), and even

studies of intracellular metabolic changes during bioethanol fermentation (Chen et al., 2016).

It was observed that all the metabolites followed an upward trend over time. In almost all cases there were significant differences between the cultures at two, three, and seven days. An increasing trend emerges when the significant differences between the number of colonies are considered individually. In all the groups of metabolites, one colony and two colonies are clustered in two different groups that are significantly different from a third group formed by three and four colonies. There was one exception: total AA were separated into four classes according to the number of colonies.

If the statistical results are analyzed together, the impact of the number of colonies considered becomes apparent mainly on the seventh day (Figure 2). For PO_4^- (Figure 2A), organic acids (Figure 2B), and trehalose (Figure 2D), there were no differences between three and four colonies on the seventh day. As with the lipid profiles, this could be due to a large quantity of these metabolites in the yeast cells during this growth period, inducing a non-linear response of the detector. This would therefore make it necessary to dilute the sample. The same happened between two and three colonies when detecting the amino acids identified. In relation to evolution over time, some differences were also found between the second and third day. However, one colony was an exception among all the clusters; this is more clearly described in Figure 3.

As can be seen in Figure 2A, the content of the phosphate anion (PO_4^-) derived from phosphoric acid in cells increased with time, but only slightly according to the number of colonies, for the reasons explained above. Inorganic phosphate is an essential nutrient for all organisms and required for basic vital needs, including the synthesis of nucleic acids, phospholipids, and cellular metabolites. It is taken up from outside the cells through several membrane transport systems and is compartmentalized in yeast vacuoles as free

phosphate and polyphosphate (Persson et al., 2003). Sommer (1996) reported that yeast extract contains ca. 1.8% (w/w), suggesting that there is no limitation of extracellular phosphate. In addition, Markham and Byrne (1967, 1968) showed that the limiting concentration of P with 5% (w/v) glucose in the growth medium was 65 μg/mL. This could mean that, under our conditions, the increasing levels of intracellular phosphate detected over time could be due to an increase in the release of phosphate from its storage form in vacuoles and hence its greater availability, or an experimental artifact related to the sampling process.

For the total organic acids (succinic, malic, fumaric and citric acids), a plateau was observed at day 3 regardless of the number of colonies analyzed; however, subsequently (on day seven), the percentage was proportional to the cell concentration (Figure 2B). Aside from lactic acid, which is produced from pyruvic acid, the other organic acids are formed in the Krebs cycle. After 2–3 days of growth on YPDA medium, the yeast cells had consumed the sugars, producing CO_2 and generating an anaerobic atmosphere, which could explain the slow formation of these acids. However, after depletion of sugars, the production of these acids increased. These aging stress conditions could influence the increasing concentration of trehalose over time in a similar way (Figure 2D). Indeed, it has been proven that the accumulation of trehalose in yeasts is a response to certain stressful conditions, such as fermentation (Wang et al., 2014).

An interesting result is the increase over time in the levels of some amino acids and amines, such as ornithine and putrescine. In yeast cells, ornithine is decarboxylated to putrescine, which is the main substrate for polyamine biosynthesis (Tabor et al., 1982). It should be noted that ornithine and putrescine, as well as other polyamines not identified in our extract but likely to have been present, appeared from the third day onward, and their increase in time correlated with actively growing yeast (Kay et al., 1980). These

metabolites are related to aging cells and as a response to the generation of reactive oxygen species (ROS) (Eisenberg et al., 2009). It must be remembered that the YPD growth medium contains amino acids due to the presence of peptone and yeast extract, and that these may be assimilated by yeast and not synthesized. To our knowledge, there is no information available on whether these products contained other polyamines, such as cadaverine, spermine, or putrescine.

In addition, other metabolites were detected with a high degree of certainty (> 80% confidence), including glycerol and inositol. There was a significant increase in inositol related to the number of days and number of colonies. This increase could arise from two different mechanisms: firstly, because these yeasts produce inositol from glucose, and secondly, because it also has inositol transporters that incorporate it from the medium (Nikawa, 1991). Inositol is an important component of several secondary messenger molecules and phospholipids (Ploier et al., 2014); their rising concentrations could therefore be due to the increase of biomass over time.

In contrast, the concentration of glycerol only presented significant differences for one colony; in fact, the percentage of this increased on the third day and significantly decreased on the seventh day. This decrease could be due to the increase in other metabolites on day 7 as the metabolite levels were calculated from an area ratio.

3.3. Effect of number of colonies and growth time on the lipid and metabolome composition of strain *Lalvin QA23*

The lipidomic and metabolomic results of the strain Lalvin QA23 samples were subjected to a principal component analysis (PCA), which used C12, C18, C16:1, C18:1, and squalene for lipid compounds and lactic acid, glycerol, total AA, PO_4^-, organic acids, and trehalose for metabolomic compounds. The resulting model had two factors that explained 82.3% of the variance. The samples visibly clustered into three groups

according to growth day: cluster 1: second day; cluster 2: third day; and cluster 3: seventh day. PC1 described the time-evolution of the major part of the organic acids, total AA, PO_4^-, trehalose, C16:1, and C18. Furthermore, similarly to the ANOVA results (Table 3), this component also separated the third cluster according to the number of colonies, due to the increase and decrease in the metabolites and lipids mentioned previously. We should also point out that there was evident separation of one colony sample from the second and third group, located near the first cluster. With respect to the second principal component, it was observed that the main contributors to the separation of clusters were C12, C18:1, glycerol, lactic acid, and to a lesser extent squalene, which allowed a good separation of cluster 2 (colonies from the second day) from the other clusters. Finally, the first group was clearly associated with C18 synthesis.

Table 3 shows that the differences between colonies, growth time, and their interaction were statistically significant in most cases. There were some exceptions with regard to the number of colonies (which had no significant effect on SFAs, UFAs, UFA/SFA and ergosterol/squalene) and the growth time effect in the case of ergosterol. It was remarkable that the effect of the number of colonies on squalene was only significant with a confidence interval of 95%.

Even though these differences were mostly significant, it was observed that the signal obtained for one colony and two days is sufficient and reproducible. Thus, the analysis is most efficient the growth time is two days, as this is the shortest growth time necessary. Furthermore, selecting just one colony could avoid possible cross-contamination on the agar plate, which would vary the results. This should also avoid the problem of signal saturation that was observed with three and four colonies in some cases.

3.4. Differentiation of different Saccharomyces *species with the method developed*

Different strains of *S. cerevisiae* (K1, BM4x4, QA23 and EC 1118) were analyzed to observe the differences between their lipid profiles and metabolomes. These were characterized under the previously optimized conditions of two days' growth, and one colony on YPDA.

The metabolome results (two days, one colony) obtained for all strains were not sufficiently different to include them in the PCA analysis. However, a variation trend can be observed between the two species. The main differences are reflected in the fatty acid profile. The strains QA23 and EC 1118 ~~(*S. bayanus)*~~ had higher concentrations of SFAs than K1 and BM4x4 ~~(*S. cerevisiae*)~~, particularly C18. On the other hand, the last strains cited had higher percentages of UFAs than the others (results not shown). This variation was even more pronounced when comparing C18:1. Even though these differences were not significant, the trend can be seen clearly in Figure 4. The four strains were separated by principal component 1 and, the SFA and C14 variables determined this discrimination. Comparing these strains of *S. cerevisiae* with three other species of *Saccharomyces* (*S. uvarum*, *S. pastorianus*, and *S. kudriavzveii*) reveals significant differences. Principal component 2 separated the *S. cerevisiae* strains from others influenced by variable UFAs (C18:1 and C16:1) (Figure 4). However, *S. uvarum* is separated from *S. pastorianus* and *S. kudriavzveii* by the principal component 1. This species presents higher SFA values, particularly C16, than the others, which had higher UFA concentrations.

The metabolomic profiles of the seven species, after two days of growth on YPDA picking one colony, were subjected to ANOVA analysis. After two days of growth, few intracellular metabolites were identified, and significant differences were only recorded for lactic acid, trehalose, and the sum of AA as well as organic acids. For the other metabolites detected, PO_4^-, glycerol, and inositol, no significant differences were observed. With respect to lactic acid and trehalose synthesis (Supplementary File SF1),

S. kudriavzevii had the lowest content of intracellular metabolites compared to the other species. This is likely to be related to the weak growth of this species at 28°C, as its optimum temperature for growth is 24°C (Arroyo-López et al., 2009). Greater levels of trehalose were accumulated by strain Lalvin QA23. The total AA percentage was higher in *S. cerevisiae* (K1 and BM4x4) and *S. uvarum* than in *S. kudriavzveii*, *S. pastorianus* and in the other *S. cerevisiae* strains, EC1118 and QA23.

3.5. Influence of the physical state of the culture medium on the lipid composition

It was very interesting to note that the state of the growing medium, liquid or solid, made a significant difference to the UFA composition in the cells of all yeast species (Figure 5). The same behavior was observed for all the other lipids determined (Supplementary File ST2). This result shows that, independently of the yeast species analyzed, SFAs are synthesized in the first moments of cell growth on a solid growth medium. One might hypothesize that the liquid growth condition is more hypoxic than the solid medium, promoting the synthesis of unsaturated fatty acids, but this is not the case.

The same behavior was observed for *S. cerevisiae* (K1 and Lalvin QA23 strains) and *S. uvarum* but not for *S. kudriavzevii* and *S. pastorianus* (Figure 5). No significant difference was found between the *S. kudriavzevii* results in the two culture conditions, liquid *vs* solid. However, for *S. pastorianus*, although there are no significant differences, the trend is reversed, with more UFAs being recorded in the solid medium than in the liquid medium.

4. Conclusions

In this study, we used gas chromatography to conduct a lipidic and metabolomic analysis of several strains of different *Saccharomyces* species, from colonies grown on agar plates. The method conditions were optimized by studying differences in terms of number of

colonies, growth time, and culture media. There were significant changes over time and in terms of the number of colonies, as in the case of the metabolome, but we did not observe any significant changes on day 2 regardless of the number of colonies. We concluded that one single colony grown for two days is the most efficient and quickest way of producing a good chromatographic signal. To our knowledge, this is the first time it has been observed that when wine yeasts are cultivated on a solid medium there is (i) greater synthesis of C18 than of C18:1, (ii) increasing temporal evolution of intracellular metabolites, and (iii) differentiation between *Saccharomyces* species on the basis of metabolomic and lipid profiles. In this study, certain strains of *S. cerevisiae* were differentiated from each other using the fatty acid profile of a colony. ~~Some manufacturers~~. Curiously, two of these strains, EC1118 and QA23 have occasionally been named by the manufacturer as *S. cerevisiae bayanus*. Moreover, despite the hybrid origin of *S. bayanus* (*S. cerevisiae* x *S. uvarum* x *S. eubayanus*) (Ono et al., 2020), its different profile from *S. cerevisiae* confirms that there are some physiological differences between these two species. The major advantage of the method developed is that it may be very useful for differentiating those and other yeast species and even other wine microorganisms grown under the same conditions, and for tracking their main metabolomic changes over time.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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Figure captions

Figure 1. Effect of time (■ 2 days, ■ 3 days, ■ 7 days) on the lipid composition (%) of strain Lalvin QA23 cells according to the number of colonies. 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 significant difference between the number of colonies using the Tukey (HSD) test at $p < 0.05$.

Figure 2. Effect of time (■ 2 days, ■ 3 days, ■ 7 days) on the main metabolomic compounds of strain Lalvin QA23 cells according to the number of colonies. (A), organic acids (B), total amino acids (Total AA) (C), and trehalose (D). Mean ± standard deviation (n=3). Different lower-case letters indicate a significant difference between the number of colonies and time using the Tukey (HSD) test at $p < 0.05$. AU, arbitrary units: height of the metabolite normalized with the height of the internal standard. Clusters were used to visualize the different groups, they are not based on statistical intervals/analysis

Figure 3. Biplot of principal component analysis (PCA) with varimax rotation of the principal identified metabolites and lipids of the Lalvin QA23 strain. Score plots of factor 1 (55.39%) against factor 2 (15.11%) where the samples were grouped in three clusters: Cluster 1: second day; Cluster 2: third day; and Cluster 3: seventh day. U, corresponds to the number of colonies; D, corresponds to the number of days.

Figure 4. Biplot of the principal component analysis (PCA) with varimax rotation of the main fatty acid composition of the cells of the *Saccharomyces* species. SFA, saturated fatty acids (sum of C14, C16 and C18); UFA, unsaturated fatty acids (sum of C16:1 and C18:1); K1 and BM4X4, *S. cerevisiae* strains (red circle); EC1118 and QA23, *S. cerevisiae* strains (blue circle); *S. kudriavzevii*, *S. pastorianus* (green circle) and *S. uvarum* (gray circle). The ellipses added to the PCA are used as visual aids to identify the different clusters, they are not based on statistical intervals/analysis.

Figure 5. Unsaturated fatty acid composition in yeast cells growing in either liquid or solid YPD. Unsaturated fatty acids (sum of C16:1 and C18:1); *S. cerevisiae* K1 strain; *S. cerevisiae* Lalvin QA23 strain; *S. kudriavzevii* strain, *S. pastorianus,* and *S. uvarum*. Different upper-case letters indicate a significant difference in UFA composition using the Tukey (HSD) test at $p < 0.05$.

Table 1. *Saccharomyces* strains used in this study.

Species	Strain commercial name or collection number	Source
Saccharomyces cerevisiae [c]	Lalvin QA23®	Lallemand [a]
Saccharomyces cerevisiae [c]	Lalvin EC 1118®	Lallemand
Saccharomyces cerevisiae	ICV K1 *Marquée*	Lallemand
Saccharomyces cerevisiae	Lalvin BM4X4	Lallemand
Saccharomyces kudriavzevii	11825	CECT [b]
Saccharomyces uvarum	1969	CECT
Saccharomyces pastorianus	1940	CECT

[a] Lallemand Inc., Montreal, Canada

[b] CECT, Spanish Type Culture Collection

[c] Labelled as *Saccharomyces cerevisiae bayanus* by the manufacturer

Table 2

Relationship between the number of sampled colonies and the number of cells/mL determined by counting the cells of the strain Lalvin QA23 at two days. Different lower-case letters indicate a significant difference ($p < 0.05$) between the number of colonies using the Tukey (HSD) test.

Number of colonies	cells/mL 10^7
1	0.75 ± 0.014^{a}
2	1.06 ± 0.085^{b}
3	1.74 ± 0.092^{c}
4	2.23± 0.014 d

Table 3

Main effects of number of colonies and growth time on YPDA and the interaction between them on the lipid composition and main metabolomic compounds of strain Lalvin QA23. Medium-chain fatty acids (MCFA), saturated fatty acids (SFA), unsaturated fatty acids (UFA), Erg/Sq, ergosterol/squalene, (n=3 independent colonies); ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

	Colony effect	Time effect	Colony x Time
MCFA	***	***	***
SFA	ns	***	***
UFA	ns	***	***
UFA/SFA	ns	***	***
Ergosterol	***	ns	***
Squalene	*	***	***
Erg/Sq	ns	***	***
Organic acids	***	***	***
Total AA	***	***	***
Trehalose	***	***	***
PO_4^-	***	***	***

Figure 1.

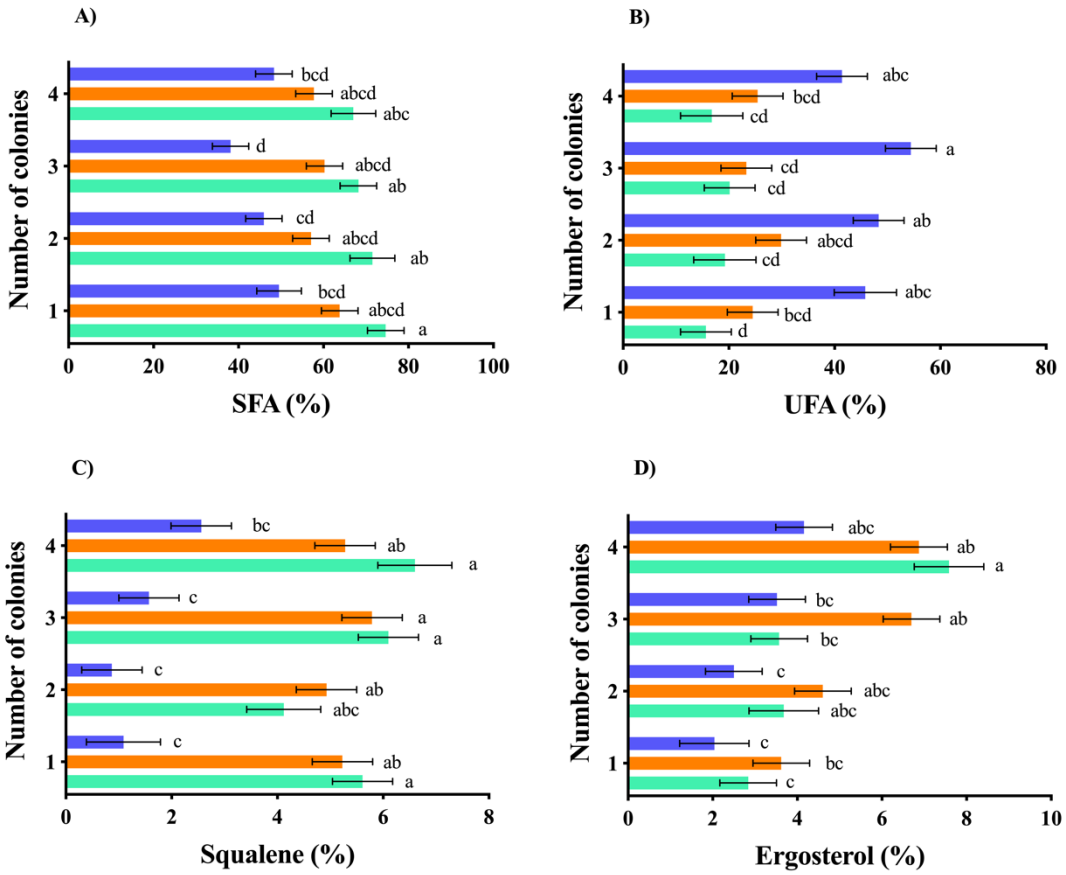


Figure 2.

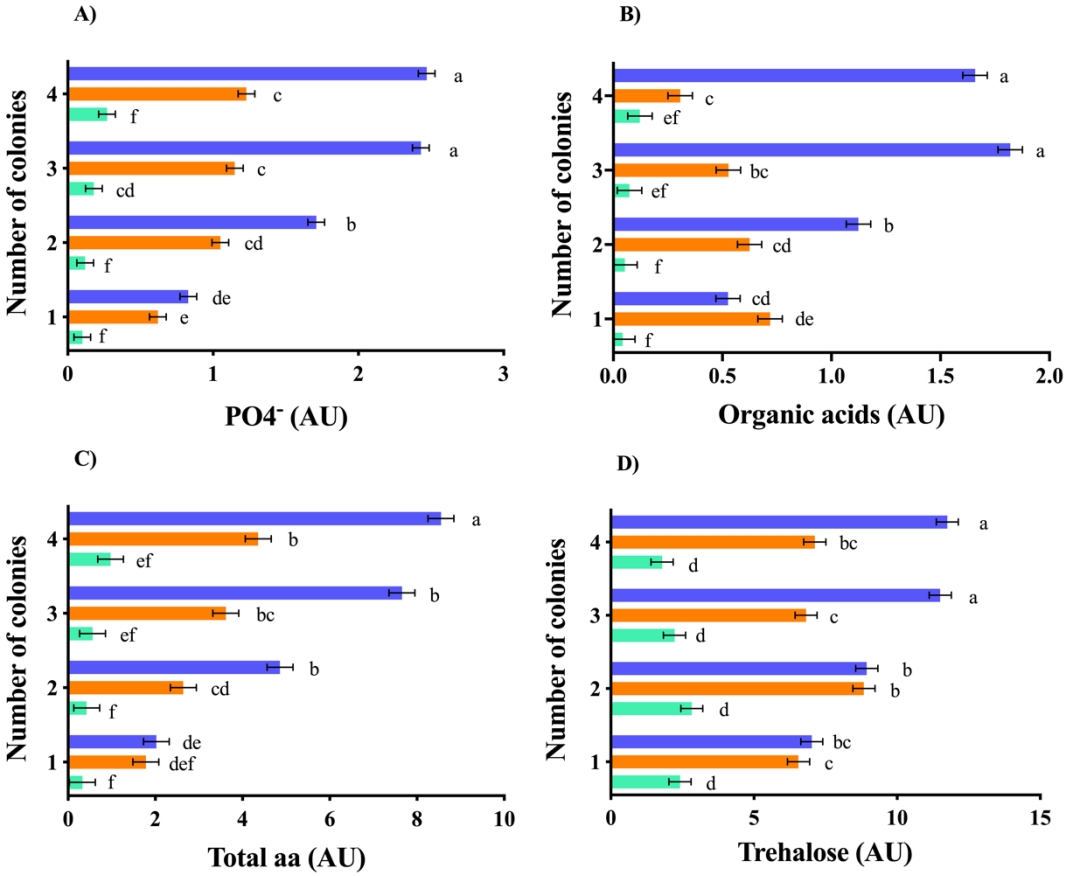
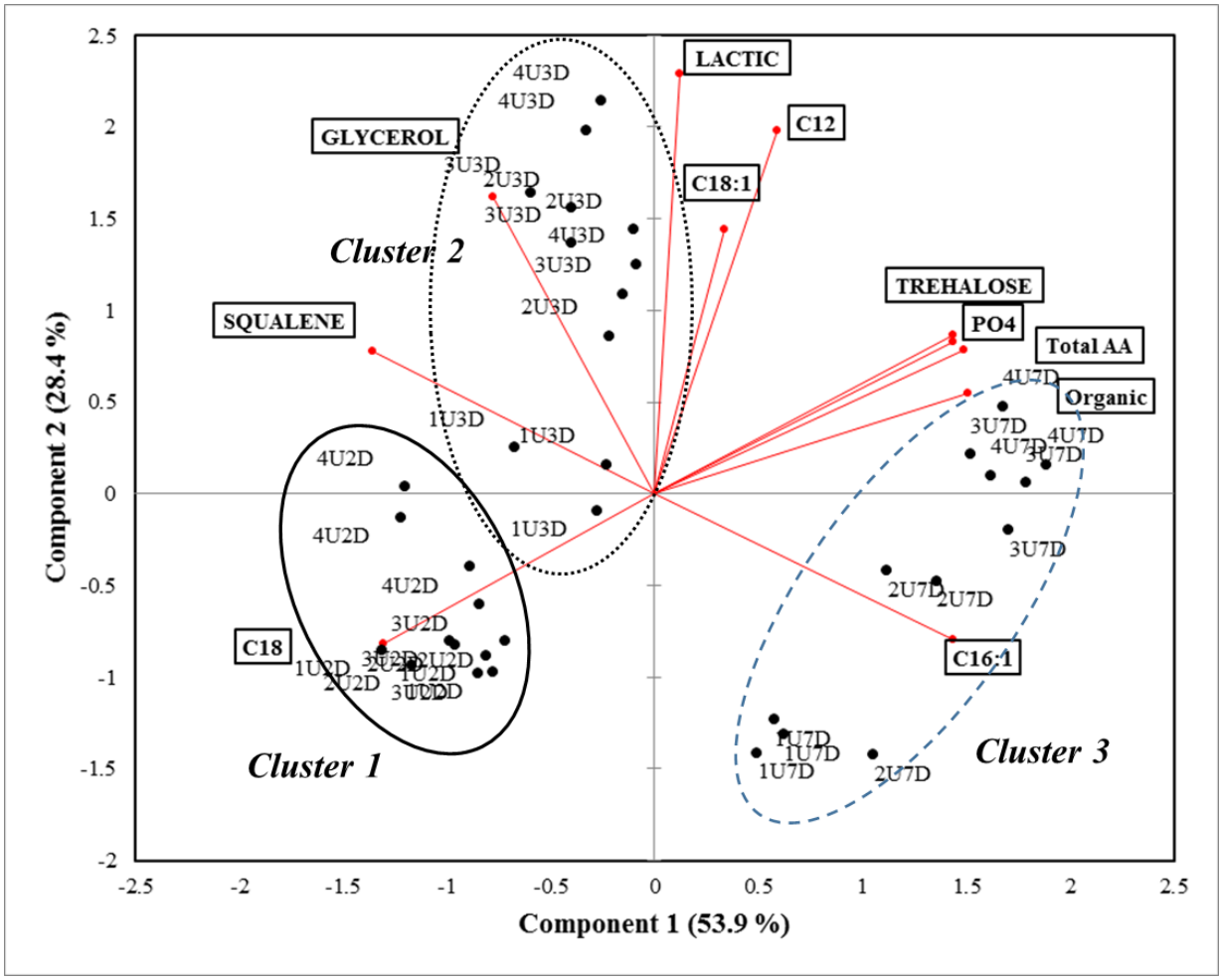
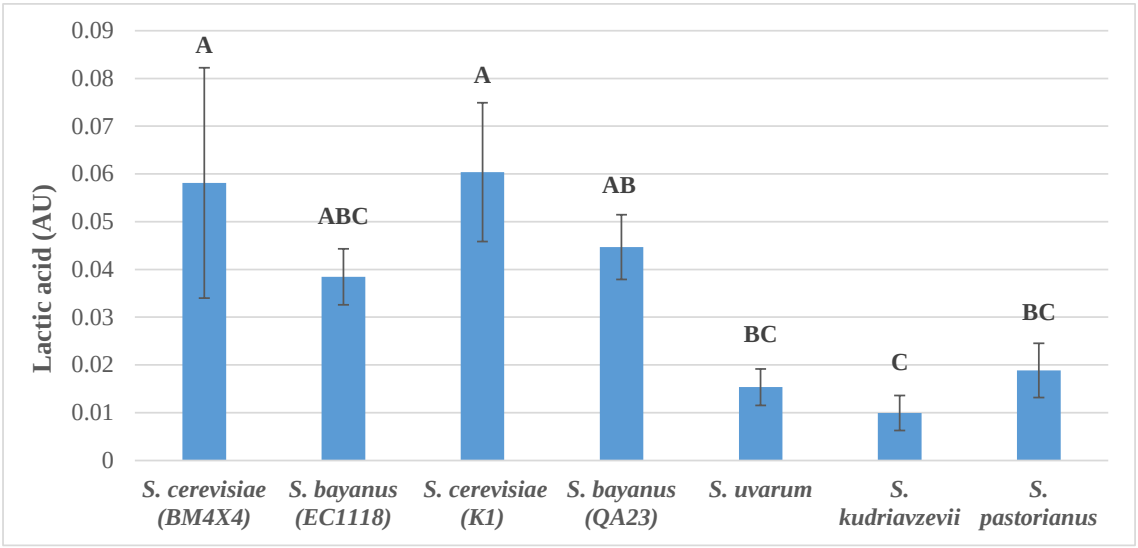


Figure 3.


LACTIC
C12
C18:1
GLYCEROL
SQUALENE
TREHALOSE
PO4
Total AA
Organic
C18
C16:1
Cluster 1
Cluster 2
Cluster 3
Component 1 (53.9 %)
Component 2 (28.4 %)

Supplementary Figure (SF1)

Effect of two days of growth on the intracellular content of lactic acid and trehalose for different *Saccharomyces* species by analysis of a single colony. Mean ± standard deviation (n=3). Different upper-case letters indicate a significant difference between *Saccharomyces* strains using the Tukey (HSD) test at $p < 0.05$. AU, arbitrary units: height of the metabolite normalized with the height of the internal standard (ribitol).



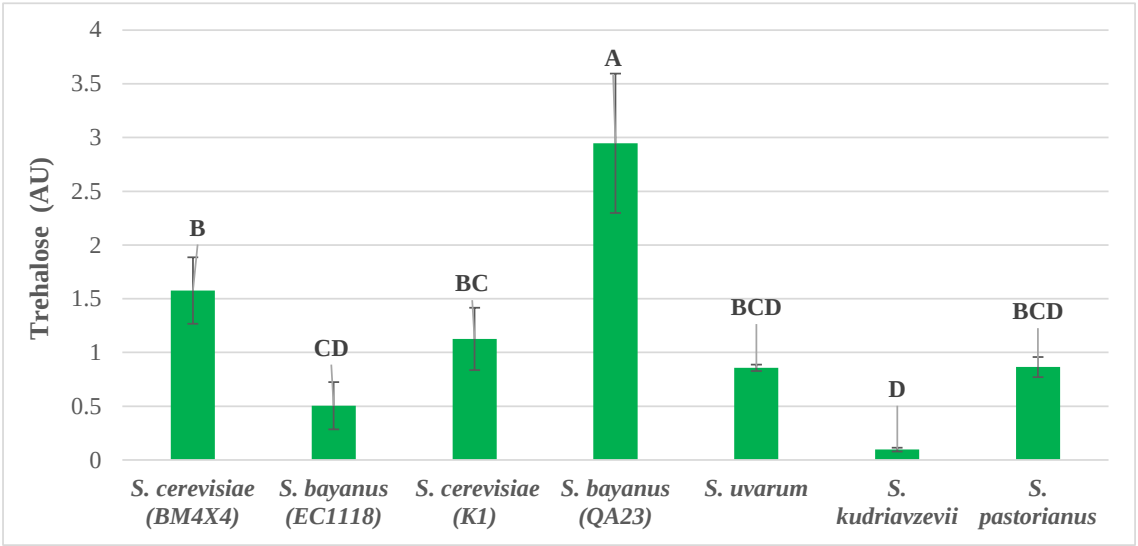


Figure 4.

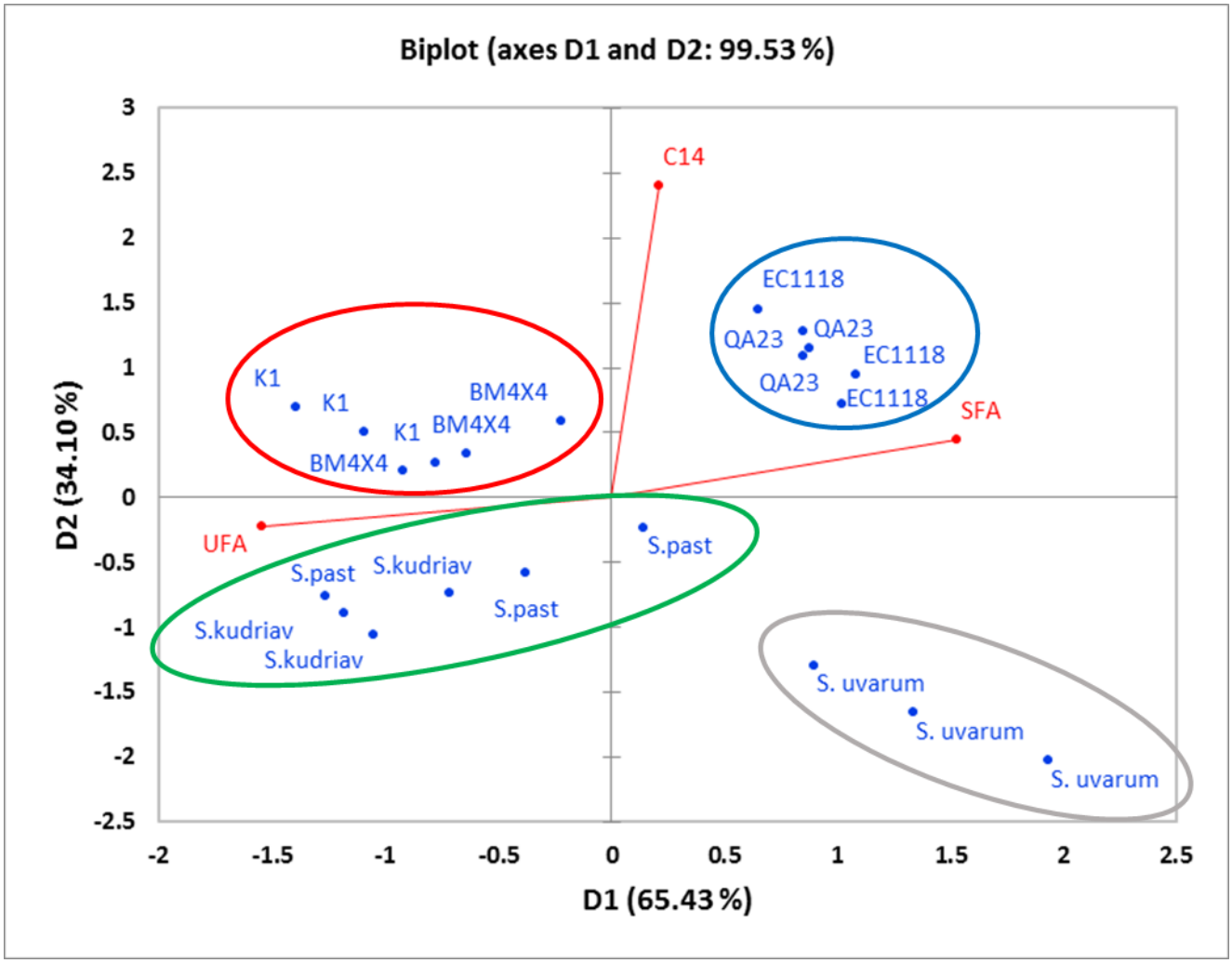
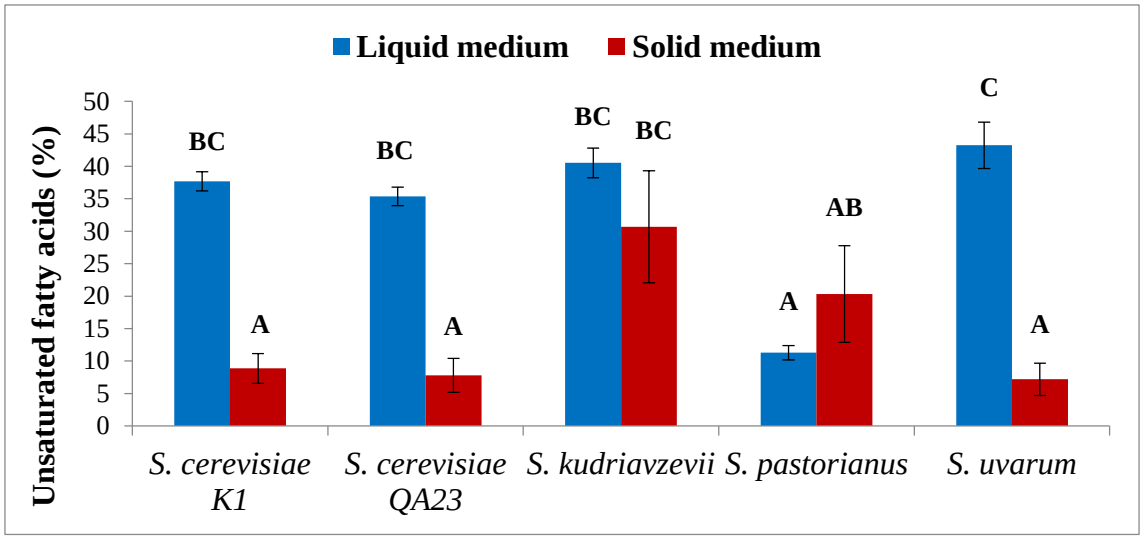


Figure 5.



Supplementary Table 2 (ST2). Influence of the physical state of the culture medium on the lipi

Medium	Species	Strains	C12	C14	C16:1
Liquid	*S. bayanus*	QA23	1.85	3.05	24.71
Liquid	*S. bayanus*	QA23	1.90	2.79	23.50
Liquid	*S. bayanus*	QA23	1.18	2.80	15.31
Liquid	*S. cerevisiae*	K1	2.70	5.26	26.69
Liquid	*S. cerevisiae*	K1	1.61	4.51	26.10
Liquid	*S. cerevisiae*	K1	1.18	2.80	15.31
Liquid	*S. kudriavzevii*	S. kud	0.95	1.84	26.68
Liquid	*S. kudriavzevii*	S. kud	1.17	1.81	27.86
Liquid	*S. kudriavzevii*	S. kud	1.34	2.30	27.99
Liquid	*S. uv arum*	S. uv	2.05	3.37	27.33
Liquid	*S. uv arum*	S. uv	2.81	5.61	30.34
Liquid	*S. uv arum*	S. uv	1.79	3.94	31.38
Liquid	*S. pastorianus*	S. past	0.94	7.85	9.70
Liquid	*S. pastorianus*	S. past	0.86	8.42	9.78
Liquid	*S. pastorianus*	S. past	1.11	5.72	10.11
Solid	*S. bayanus*	QA23	0.78	1.31	3.07
Solid	*S. bayanus*	QA23	0.00	1.44	3.68
Solid	*S. bayanus*	QA23	0.00	1.61	2.65
Solid	*S. cerevisiae*	K1	0.81	0.85	4.42
Solid	*S. cerevisiae*	K1	0.93	0.85	5.30
Solid	*S. cerevisiae*	K1	1.03	0.97	3.13
Solid	*S. kudriavzevii*	S. kud	0.00	1.73	14.46
Solid	*S. kudriavzevii*	S. kud	0.00	2.12	22.79
Solid	*S. kudriavzevii*	S. kud	0.00	1.51	15.51
Solid	*S. uv arum*	S. uv	1.05	0.99	3.20
Solid	*S. uv arum*	S. uv	0.65	1.23	6.08
Solid	*S. uv arum*	S. uv	5.46	1.51	7.59
Solid	*S. pastorianus*	S. past	0.00	2.40	8.00
Solid	*S. pastorianus*	S. past	0.00	1.89	11.18
Solid	*S. pastorianus*	S. past	1.10	1.60	15.31

d composition (%)

C16	C18:1	C18	Squalene	Ergosterol	SFA
33.86	11.66	17.68	4.20	2.99	56.43
34.37	10.85	17.31	4.65	4.62	56.38
39.57	8.12	29.29	1.60	2.14	72.84
29.89	12.05	13.63	5.24	4.55	51.47
33.00	10.55	15.54	4.53	4.16	54.66
39.57	8.12	29.29	1.60	2.14	72.84
34.20	13.26	16.81	3.35	2.91	53.80
42.28	10.72	13.93	1.01	1.22	59.19
31.53	15.07	17.29	2.15	2.33	52.46
35.24	12.08	14.92	2.56	2.46	55.58
32.97	13.49	11.08	1.96	1.73	52.48
32.87	15.12	12.58	1.24	1.07	51.19
43.21	0.66	34.30	1.71	1.61	86.31
41.02	2.73	33.07	1.25	2.87	83.37
42.82	0.84	34.46	1.68	3.25	84.12
42.62	4.38	42.75	2.22	2.88	87.46
43.75	6.91	40.68	1.62	1.91	85.87
46.96	2.76	45.13	0.89	0.00	93.71
42.65	5.28	43.59	1.40	1.01	87.90
43.52	5.34	41.60	1.67	0.79	86.90
48.21	3.12	43.53	0.00	0.00	93.74
36.94	9.79	35.21	0.00	1.86	73.89
51.25	17.71	3.14	0.00	3.00	56.51
35.64	11.77	30.96	0.00	4.61	68.11
49.23	1.08	44.44	0.00	0.00	95.72
38.00	2.54	50.99	0.00	0.51	90.87
45.41	1.04	37.06	1.39	0.54	89.44
43.45	4.38	41.52	0.00	0.25	87.37
38.45	10.21	36.31	0.00	1.96	76.65
34.64	11.85	30.55	0.00	4.94	67.90

UFA
36.37
34.35
23.42
38.74
36.65
23.42
39.94
38.58
43.06
39.41
43.83
46.50
10.37
12.51
10.95
7.45
10.59
5.40
9.69
10.64
6.26
24.25
40.50
27.28
4.28
8.62
8.63
12.39
21.39
27.16

Supplementary Table 1 (ST1). Effect of time (2, 3, and 7 days) and number of colonies (1, 2, internal standard (ribitol for metabolites and cholestane for lipids).

Condition	TIME	NUMBER	PO4	*GLYCEROL*	***LACTIC***	SUCC
1UFC 2Days	2 DAYS	1COL	0.09961983	0.11129192	0.01264328	0.02622418
1UFC 2Days	2 DAYS	1COL	0.07924459	0.08627019	0.01433924	0.01579365
1UFC 2Days	2 DAYS	1COL	0.12154712	0.13578835	0.01542619	0.03199638
2UFC 2Days	2 DAYS	2COL	0.12761725	0.19246904	0.01422841	0.03100682
2UFC 2Days	2 DAYS	2COL	0.1153789	0.12889742	0.01464335	0.03037264
2UFC 2Days	2 DAYS	2COL	0.12360467	0.17162588	0.01436446	0.03193474
3UFC 2Days	2 DAYS	3COL	0.16318189	0.09441148	0.02008269	0.03771223
3UFC 2Days	2 DAYS	3COL	0.21917657	0.12955864	0.02298737	0.05024505
3UFC 2Days	2 DAYS	3COL	0.17250893	0.13971546	0.01693679	0.04470796
4UFC 2Days	2 DAYS	4COL	0.21631976	0.147771	0.02140789	0.0604878
4UFC 2Days	2 DAYS	4COL	0.32467757	0.22803542	0.03568457	0.09248436
4UFC 2Days	2 DAYS	4COL	0.26543234	0.26820044	0.02787872	0.06984468
1UFC 3Days	3 DAYS	1COL	0.58742905	0.14356534	0.06703024	0.23746175
1UFC 3Days	3 DAYS	1COL	0.67394135	0.11313679	0.0705925	0.25397535
1UFC 3Days	3 DAYS	1COL	0.59668659	0.12483791	0.07403046	0.19194954
2UFC 3Days	3 DAYS	2COL	1.0511119	0.24676593	0.15870309	0.41752562
2UFC 3Days	3 DAYS	2COL	1.05247504	0.18563513	0.09155946	0.379572
2UFC 3Days	3 DAYS	2COL	1.05181191	0.21537383	0.12422326	0.39803555
3UFC 3Days	3 DAYS	3COL	1.13820527	0.23790154	0.13876957	0.49760886
3UFC 3Days	3 DAYS	3COL	1.12290237	0.17084359	0.13513825	0.49697889
3UFC 3Days	3 DAYS	3COL	1.18892804	0.24905167	0.11802679	0.48809193
4UFC 3Days	3 DAYS	4COL	1.12177756	0.26290648	0.12783915	0.55423855
4UFC 3Days	3 DAYS	4COL	1.28937581	0.20066875	0.17968537	0.59649481
4UFC 3Days	3 DAYS	4COL	1.28590748	0.17829099	0.18789485	0.52045911
1UFC 7Days	7 DAYS	1COL	0.84171244	0.04358834	0.01417533	0.38025738
1UFC 7Days	7 DAYS	1COL	0.68442477	0.02224351	0.00922636	0.32060344
1UFC 7Days	7 DAYS	1COL	0.95795127	0.02943656	0.01096606	0.42640637
2UFC 7Days	7 DAYS	2COL	1.50814945	0.0456176	0.02812254	0.68591596
2UFC 7Days	7 DAYS	2COL	1.98478992	0.06987687	0.03260335	1.0764123
2UFC 7Days	7 DAYS	2COL	1.64353577	0.06705085	0.04278163	0.76044593
3UFC 7Days	7 DAYS	3COL	2.39657629	0.12580315	0.05979078	1.26913037
3UFC 7Days	7 DAYS	3COL	2.53777909	0.13276056	0.03085162	1.48127039
3UFC 7Days	7 DAYS	3COL	2.35696748	0.08378661	0.04196618	1.30749796
4UFC 7Days	7 DAYS	4COL	2.43735656	0.05947653	0.09405075	1.15725302
4UFC 7Days	7 DAYS	4COL	2.60598926	0.14418047	0.08770707	1.36314537
4UFC 7Days	7 DAYS	4COL	2.358439	0.08796974	0.07625581	1.1249313

, 3, and 4) on the major identified metabolomic and lipid compounds of QA23 yeast cells. /

FUMARIC	MALIC	CITRIC	Organic	ALA	ETHYLPRO	VAL
0.00229669	0.00937731	0.00596335	0.04386154	0.01086007	0.00614798	0.0122399
0.00201496	0.00797659	0.00621507	0.03200028	0.01366351	0.00303969	0.01442189
0.00228407	0.01144135	0.00727595	0.05299774	0.01325047	0.00750121	0.01493403
0.00317153	0.01122114	0.00890754	0.05430702	0.01954026	0.00541705	0.01594749
0.00216816	0.01086073	0.00690671	0.05030824	0.01257804	0.00712054	0.01417616
0.00284255	0.01110297	0.00825153	0.05413179	0.01725756	0.00597557	0.01536673
0.00201734	0.01403586	0.00958205	0.06334747	0.03194466	0.00616607	0.01659161
0.00160819	0.01798676	0.01166348	0.08150348	0.01559626	0.01012299	0.01311266
0.00401669	0.01914515	0.00863354	0.07650334	0.0212548	0.01579591	0.0161857
0.00422829	0.02377923	0.01078841	0.09928373	0.04983887	0.01139281	0.01686109
0.00636754	0.03576634	0.0155496	0.15016784	0.05460847	0.01212453	0.02528768
0.00519789	0.02921232	0.01294639	0.11720129	0.05200067	0.01172446	0.0206804
0.00993028	0.04653365	0.02027502	0.3142007	0.03586499	0.07308762	0.02221011
0.01472961	0.05439817	0.01959908	0.34270221	0.05108837	0.06122558	0.02939047
0.00947525	0.04241917	0.01962258	0.26346654	0.06652624	0.0313718	0.01724799
0.016026	0.07932594	0.03329252	0.54617007	0.05429278	0.12293415	0.0306949
0.01695713	0.08521217	0.02765198	0.50939328	0.11393486	0.09078347	0.00394963
0.01650416	0.08234866	0.03039597	0.52728433	0.08492039	0.10642402	0.01696058
0.01754155	0.07232952	0.03773714	0.62521707	0.2075781	0.20159135	0.08323523
0.02198652	0.07929496	0.03644526	0.63470563	0.13889397	0.17618463	0.08547271
0.01796005	0.07464118	0.03399168	0.61468484	0.20426072	0.13860512	0.07860243
0.0214904	0.08912123	0.04665699	0.71150717	0.21729805	0.21959046	0.0964434
0.02274386	0.08741362	0.04366425	0.75031654	0.12861859	0.02350517	0.0340068
0.02200071	0.10781578	0.04557488	0.69585048	0.31580146	0.11973922	0.00293716
0.02295188	0.12123225	0.02030506	0.54474657	0.08744346	0.11773836	0.09142693
0.01969016	0.08968449	0.01998356	0.44996164	0.03581581	0.10303822	0.01718231
0.01881366	0.11143649	0.02566046	0.582317	0.08957727	0.15386119	0.02415122
0.0442217	0.18577069	0.0439764	0.95988475	0.08131919	0.28558881	0.11451053
0.04889842	0.22336497	0.05389147	1.40256716	0.11272015	0.44793773	0.22889128
0.03183552	0.17451401	0.04276728	1.00956274	0.14496356	0.26694867	0.12824113
0.07078045	0.3044502	0.07640573	1.72076675	0.1297667	0.55476231	0.16982102
0.07735749	0.30485012	0.09648621	1.95996421	0.37732237	0.52571285	0.29982171
0.07138806	0.3260206	0.0741054	1.77901201	0.26472188	0.4204129	0.26950164
0.06997593	0.27712454	0.07994148	1.58429496	0.30689241	0.43618951	0.19962766
0.07500896	0.30665486	0.10640429	1.85121348	0.34055087	0.48278718	0.30817029
0.06240208	0.27034448	0.08238243	1.54006029	0.21438622	0.38131468	0.22787801

AU, arbitrary units: height of the metabolite normalized to the height of the

SER1	LEU	PRO+ILE	THR1	GLY	*SER*	**THR**
0.00158353	0.0059784	0.00605798	0.01647768	0.01625701	0.00292483	0.00228282
0.01329799	0.00546865	0.00765934	0.01457439	0.01832483	0.00290849	0.0023276
0.00193208	0.0072943	0.00739139	0.02010458	0.01983534	0.00305881	0.0024126
0.01934317	0.00968724	0.01345004	0.02661075	0.02037838	0.00330489	0.00378898
0.00183403	0.00692414	0.0070163	0.01908432	0.01882875	0.00338752	0.00229017
0.01360247	0.0087813	0.01134062	0.02414307	0.0198703	0.00333198	0.00329757
0.02446724	0.01320334	0.01650587	0.0255846	0.02740151	0.01009045	0.014903
0.0342682	0.00475485	0.00264109	0.0390908	0.02698743	0.0018772	0.00168079
0.00287608	0.00548625	0.00531619	0.04028157	0.02473577	0.00399965	0.00463998
0.03286535	0.01883376	0.02495036	0.04795759	0.03340625	0.01514446	0.02018883
0.00399885	0.01877625	0.01675284	0.06906223	0.04959545	0.01245104	0.0139007
0.01978177	0.0188077	0.02123488	0.05752315	0.04751022	0.01392368	0.01733877
0.10538543	0.01109581	0.00717556	0.10805123	0.06652835	0.00875949	0.01463103
0.10176089	0.0133901	0.00968096	0.10905274	0.07299367	0.00333275	0.00771346
0.0660754	0.07501119	0.01856625	0.08173733	0.01172396	0.0061108	0.00414472
0.15110625	0.02414526	0.01049757	0.16180518	0.09938452	0.00947255	0.01752148
0.12590852	0.07475919	0.0255979	0.16156556	0.10930815	0.00263842	0.00690263
0.13816662	0.0501367	0.01825195	0.16168213	0.10448054	0.00596306	0.01206845
0.23379781	0.0254579	0.01747569	0.22745744	0.0132933	0.05421632	0.06895386
0.20636684	0.02315914	0.02248868	0.20082914	0.04194294	0.051873	0.02419937
0.25647832	0.02588327	0.03566082	0.18406056	0.04522134	0.06051612	0.02092318
0.22889477	0.03280757	0.02137518	0.22264202	0.04722993	0.08976255	0.09412173
0.24993354	0.03172433	0.0212862	0.25961759	0.16754223	0.01132509	0.01486185
0.18036592	0.3261823	0.1134506	0.21628913	0.24663209	0.0435994	0.01399318
0.09198668	0.01702155	0.0252952	0.10162609	0.07411091	0.01457134	0.00443709
0.07528106	0.02144156	0.02021934	0.0708205	0.00773014	0.01898659	0.00173883
0.09631738	0.0222716	0.03254906	0.10930142	0.03245626	0.01204444	0.00284117
0.18277324	0.04395956	0.03752448	0.22507655	0.08766265	0.01743305	0.00454364
0.26016074	0.06409851	0.06575506	0.27276316	0.12386201	0.01612799	0.00573766
0.18868963	0.0539219	0.06619103	0.22789163	0.14766084	0.02434295	0.00738892
0.30252246	0.08288547	0.10106415	0.35791947	0.14955098	0.01637516	0.00835961
0.33399977	0.08837918	0.12177622	0.45156032	0.20291089	0.01528715	0.0086994
0.30011812	0.07374951	0.09433888	0.39789051	0.18486762	0.0240658	0.00915271
0.244107	0.07666149	0.10945048	0.35528674	0.20625969	0.01347352	0.01366174
0.3163356	0.09577612	0.12717128	0.49344758	0.19240387	0.01372223	0.01169725
0.26793343	0.07272018	0.13985861	0.3309981	0.1632577	0.02533129	0.00922107

OXOPRO	**ASP**	GABA	PHE	GLU	HOMOCYS	ASN
0.00351269	0.04396628	0.04396628	0.00673491	0.08023301	0	0.00582798
0.03277144	0.03745754	0.03745754	0.00417137	0.06273153	0	0.00480681
0.00428587	0.05364369	0.05364369	0.00821733	0.09789307	0	0.00711078
0.05096852	0.04118639	0.04118639	0.01117603	0.13537891	0	0.00985076
0.00406837	0.0509214	0.0509214	0.00780032	0.09292523	0	0.00674992
0.03559142	0.0443782	0.0443782	0.01006924	0.12145967	0	0.00883409
0.07269799	0.03858212	0.03858212	0.02322108	0.19390715	0	0.01521314
0.01129753	0.00173555	0.00173555	0.0013308	0.1724138	0	0.01313459
0.00416643	0.05118989	0.05118989	0.02254298	0.18738255	0	0.0150369
0.09273206	0.05410074	0.05410074	0.04021067	0.24474781	0	0.02554397
0.00438701	0.08882114	0.08882114	0.03659633	0.30474982	0	0.0287094
0.09923976	0.06983757	0.06983757	0.03857249	0.27194339	0	0.02697868
0.27471513	0.23487242	0.27442898	0.02988884	0.22630674	0.00274179	0.01670457
0.32529269	0.26082268	0.26016509	0.02047024	0.1937057	0.00315129	0.01559877
0.29915957	0.2146612	0.25999133	0.00907547	0.04103213	0.00271911	0.00592872
0.46800652	0.0095124	0.37179914	0.06076417	0.45155482	0.00487968	0.03951965
0.51765688	0.01335436	0.42512404	0.02554743	0.34471157	0.00534581	0.00936939
0.49350314	0.01148534	0.39918273	0.04267955	0.3966883	0.00511905	0.02403678
0.50532252	0.01916976	0.60754902	0.10555083	0.67117868	0.00795257	0.07400807
0.56210937	0.01240654	0.54893446	0.08816129	0.54190688	0.0076491	0.07577021
0.44877657	0.03007597	0.64122132	0.12507851	0.53536193	0.00650918	0.09446162
0.58154674	0.01213835	0.68239357	0.15479441	0.82970864	0.01311194	0.123121
0.67016526	0.01416575	0.61892018	0.10402965	0.66915834	0.00831105	0.09480734
0.6986445	0.02557754	0.64113514	0.15066395	0.7936634	0.01050757	0.09901884
0.10873	0.011043	0.61018165	0.02005106	0.07668094	0.00691655	0.01589941
0.0686921	0.00401205	0.57625193	0.01179242	0.03806532	0.00522809	0.00667074
0.12862083	0.00680104	0.60284025	0.02605336	0.07017497	0.00870693	0.0133379
0.18103678	0.0215082	1.17120081	0.05281238	0.15943851	0.00711952	0.04545566
0.25583748	0.02049248	1.44905165	0.050248	0.20906086	0.006453	0.04270644
0.23907515	0.02552491	1.24031493	0.1349814	0.27272766	0.00804635	0.09297318
0.31028569	0.0179057	1.78474336	0.07322887	0.32243485	0.02609178	0.06536164
0.41218892	0.04305358	2.15417385	0.11597896	0.6067149	0.0114908	0.12838523
0.34366874	0.02926968	1.53980556	0.13260498	0.48228022	0.01322458	0.11205194
0.35675232	0.04663534	1.84966172	0.3098684	0.65454009	0.01359729	0.16766565
0.54312782	0.06666021	2.01267317	0.15769046	0.78905607	0.02019334	0.17553112
0.42514844	0.04797896	1.64475832	0.24520202	0.56147133	0.01795731	0.13439332

CITRU	GLN	ORN	***PUTRE***	HIS	TYR	CADAV
0	0.00236322	0.01521161	0	0.01031875	0.00754273	0
0	0.00162882	0.00904371	0	0.00625339	0.0065977	0
0	0.00215545	0.01855983	0	0.01259001	0.00920296	0
0	0.0033171	0.02338914	0	0.01005334	0.0094508	0
0	0.00204606	0.01761796	0	0.0119511	0.00873594	0
0	0.00290037	0.02149695	0	0.01067555	0.00921642	0
0	0.00470206	0.04106434	0	0.02235141	0.01494195	0
0	0.00523568	0.03461426	0	0.02561001	0.03418671	0
0	0.00484428	0.02856132	0	0.02514144	0.01377877	0
0	0.01118293	0.04030344	0	0.03842212	0.02110132	0
0	0.01162891	0.0570156	0	0.05551702	0.02575478	0
0	0.01138506	0.04787813	0	0.04617029	0.02321047	0
0.00704058	0.02130092	0.05979801	0.07718521	0.07418169	0.05671948	0.00425981
0.00744598	0.02322465	0.06217424	0.11420049	0.06851477	0.04091463	0.00564429
0.00815605	0.02540357	0.01037862	0.3258484	0.00783504	0.01158133	0.00437958
0.00929767	0.03514911	0.09126382	0.12224128	0.13088199	0.08631416	0.00802339
0.01412894	0.03902735	0.03551276	0.4275024	0.01563435	0.03263903	0.00835366
0.01177864	0.03714068	0.06263434	0.27900003	0.07169962	0.05875072	0.00819299
0.00514093	0.03458788	0.1753344	0.10555313	0.20240694	0.07320005	0.01006638
0.00953083	0.04829415	0.14858873	0.14971163	0.17744513	0.08583862	0.01269989
0.00649836	0.03183919	0.16513456	0.13772177	0.1701197	0.06899798	0.01900963
0.00812436	0.05296465	0.21354253	0.06305163	0.20837196	0.13509998	0.01221382
0.011424	0.0607522	0.002692	0.21975681	0.18245281	0.1112419	0.01499334
0.01462138	0.0629547	0.18967431	0.255195	0.182569	0.12003313	0.01584002
0.01015441	0.0205295	0.10373083	0.11861123	0.05710418	0.04282551	0.0064181
0.00559154	0.00482035	0.02550204	0.19550239	0.03677074	0.03525926	0.00450245
0.00928282	0.01291078	0.05428339	0.20550999	0.08285799	0.05904293	0.01015327
0.00913252	0.0623761	0.18382114	0.19617553	0.17100269	0.12158845	0.00777595
0.01128818	0.03437823	0.11203067	0.47038735	0.18553545	0.1245148	0.01334465
0.01252106	0.07568859	0.28967966	0.13878837	0.23514832	0.14007218	0.01394424
0.01205087	0.12413093	0.19363267	0.39509005	0.333214	0.19621932	0.01578429
0.01275433	0.16841931	0.35509611	0.50862105	0.38020276	0.27515283	0.01842414
0.01380274	0.10072223	0.35589682	0.25985332	0.37595306	0.22173781	0.0163926
0.01645942	0.20388055	0.51427242	0.23600983	0.38422524	0.2738877	0.02182047
0.01363443	0.18840857	0.42236767	0.39424644	0.5017152	0.34417252	0.02365157
0.01406298	0.16936914	0.40087036	0.25072576	0.36315599	0.21947265	0.01876713

LYS	TRP	Total AA	Total Amines	Total Acids	MYOINOSITOL	TREHALOSE
0.00442642	0.0040332	0.3089473	0.02782923	0.16816557	0.00573953	2.16395072
0.00364343	0.0031857	0.30543537	0.01912277	0.13764662	0.00590321	2.45028957
0.00540072	0.00492095	0.37533917	0.03322677	0.20518045	0.00700286	2.64025732
0.00241966	0.00495648	0.48080176	0.03897666	0.21775169	0.00840162	3.08792919
0.00512665	0.00467122	0.35677554	0.03154059	0.19476804	0.00664748	2.50627061
0.0033072	0.00486295	0.44013743	0.03653861	0.21021606	0.00782649	2.89722146
0.0055706	0.00906623	0.66675852	0.06655014	0.27107139	0.01184802	2.88994068
0.00577198	0.00527567	0.46247442	0.05875652	0.17588491	0.01357457	1.58155674
0.0063552	0.00473866	0.5555002	0.05479769	0.28976233	0.01042662	2.20764556
0.01176469	0.0100674	0.91571725	0.08879503	0.35294928	0.01772378	2.39945013
0.01352035	0.00896999	1.00104953	0.11087425	0.4823921	0.02084051	1.14598545
0.01225605	0.00957	1.00740516	0.09849793	0.41161853	0.01913642	1.83132415
0.00913609	0.01983376	1.84190363	0.19542518	0.73560814	0.04045438	6.86940028
0.00816179	0.01424259	1.88335891	0.23645022	0.71469347	0.04114161	6.65309038
0.00758277	0.01323219	1.62548075	0.38767771	0.51568466	0.0383201	6.15119422
0.01288925	0.03479437	2.61874605	0.31838416	0.83286636	0.0761606	9.10257132
0.0182257	0.01819857	2.66568056	0.5521202	0.78318997	0.05702552	8.58226394
0.01562964	0.02627204	2.64284802	0.4384131	0.80735636	0.06633428	8.83538126
0.01144005	0.04382235	3.78534057	0.41613085	1.29789746	0.10521792	6.9777346
0.01022843	0.03932213	3.4900078	0.45482386	1.10324789	0.0845796	7.02240823
0.01631121	0.03021854	3.57754793	0.47097634	1.20665922	0.10550068	6.46335034
0.01045193	0.05166795	4.42246915	0.48346993	1.52424056	0.10122486	6.63231694
0.01100984	0.03955312	3.77585497	0.41543553	1.30224427	0.10590163	7.60746676
0.01118485	0.0308327	4.88110649	0.64848911	1.46037607	0.12357568	7.13586463
0.31807994	0.02813435	2.18074828	0.59342343	0.69790559	0.02315722	7.41631609
0.21296289	0.01965124	1.62352992	0.45555239	0.6183293	0.02648675	6.57195682
0.36721942	0.03696239	2.27012928	0.67269756	0.67981626	0.03301436	7.07717642
0.69825142	0.05018908	4.21927643	1.20298832	1.35214752	0.06855884	8.00697222
0.63603695	0.06293389	5.28235438	1.32017248	1.678605	0.0928951	10.1851518
0.85400964	0.0513137	5.08104957	1.47760473	1.5385675	0.07452103	8.62951423
0.89103497	0.07826988	6.71250621	1.69708542	2.12508391	0.10582264	11.4732064
1.27959439	0.09226635	8.98798737	2.47129456	2.80394233	0.12478294	12.1975558
1.15367094	0.07863526	7.26839006	2.0123906	2.05135546	0.10074555	10.85296
1.39368629	0.09011246	8.49468544	2.55379463	2.55083715	0.13958908	10.9724448
1.41767538	0.10119556	9.55406177	2.63551518	2.86838944	0.1419968	13.3741023
1.16934165	0.08591552	7.60149019	2.15753034	2.25420862	0.11556465	10.9187369

C10	***C12***	*C14*	C16:1	***C16***	***C18:1***	C18
0.76662537	1.20360678	1.82208395	8.812697	49.8485281	6.52378411	24.7011338
0	0	3.42205584	5.28661695	38.0504249	6.47702158	36.1268068
0.69468015	0.9633195	1.80478609	11.2747051	44.6456146	8.67579642	23.5360706
0	0.95527022	2.97045664	9.12883374	29.700547	6.8113727	43.5327318
0.77173451	1.10444098	1.72623955	12.9549349	44.3406711	9.5965117	20.8018946
0.69841555	1.06992996	3.64986414	12.6069846	32.5533277	9.99136209	32.0305982
0.86135065	1.22318493	1.86247735	12.7043039	41.9118776	9.42580894	21.642886
0.82315697	1.15784871	1.93788427	14.9270468	46.4789438	0.82681863	22.6077739
0.95267377	1.169791	1.69108343	13.5469377	39.361962	9.82983348	18.6378524
0.88792442	0.9949092	1.70038022	0.42216497	48.1318784	9.71648428	24.583278
1.27020461	1.57570376	2.05243764	19.7157099	37.3752927	13.8237761	16.7614872
1.30688282	1.34912672	2.09276927	16.42325	39.2522843	11.7171037	18.3474499
1.32064984	1.55234503	2.4542329	0.92632224	51.2357266	10.9834116	21.9052308
2.12458486	2.02703995	2.49858795	21.3465557	36.1142868	13.6957432	14.3273007
0.96523874	1.23337263	2.613806	14.0966312	33.379646	22.6534014	14.6602546
2.09028152	2.16194325	2.69262908	1.1065804	46.4558723	16.7149809	18.4425776
2.17762561	1.65177246	2.16375816	20.7906701	35.1436054	12.8106433	14.5733598
1.95976593	1.92795134	2.65451261	1.26985645	43.7509283	16.6365962	18.6059348
2.1568863	1.91455368	2.72180456	1.23908481	43.4329861	17.22552	17.7193899
2.19349683	1.777075	2.13263904	21.6181705	34.4263016	14.6070021	13.0778678
2.66997037	2.15734369	2.61791554	1.50734909	42.7103888	17.8201028	16.0158965
2.75045366	2.38905876	2.75655733	1.3036261	42.6395571	19.4408426	16.9236048
0.47042946	0.98655788	2.144	30.9473551	31.581958	14.6010934	16.1768605
0.56272397	1.00372419	2.22833332	32.9409894	31.3652386	13.1454028	15.5944593
0.7182285	1.2903607	2.71645505	41.3616632	31.9051741	2.3194096	16.2646968
0.90992696	1.39732098	2.54211288	35.463851	28.8057612	16.0937018	11.4178133
0.80793596	1.79852121	2.98711885	36.674029	27.3898933	13.1216605	13.8928616
1.17271566	1.6812815	2.52548903	37.5164149	28.6672167	14.8471441	7.88279072
0.84307605	1.40532339	2.27471657	36.3734542	28.5657	17.9110842	8.53487403
0.582259	1.33922191	2.08479829	38.587876	27.0243468	18.0827094	6.8322349
1.17003202	2.16906967	3.00238872	37.6388162	27.8592241	14.5245762	8.13933713
1.43335049	1.99065149	3.01888861	45.5240112	32.4013048	1.81528866	7.65059144
1.35446605	2.46224769	4.46590603	0.30832737	43.9284328	24.4025338	14.5775223

SQUALENE	**ERG**	SFA	UFA
3.87267444	2.44886642	76.3717459	15.3364811
8.398213	2.2388609	77.5992876	11.7636385
4.57351663	3.83151096	69.9864713	19.9505015
3.83958896	3.06119896	76.2037354	15.9402064
4.40695501	4.29661774	66.8688052	22.5514466
		0	0
6.79676605	0.60275173	68.23379	22.5983467
5.64576845	4.72234219	65.4172409	22.1301129
5.85262794	5.38789886	71.024602	15.7538655
7.26471536	7.54515076	59.6908979	23.3767712
5.93449874	7.62848181	74.4155366	10.1386492
		0	0
4.67528357	2.75010457	56.1892175	33.539486
5.1201174	4.39101586	59.6925034	28.1403538
5.9025971	3.71948385	75.5951904	11.9097338
4.13737977	3.72852101	52.9401755	35.0422989
5.78324779	4.61440165	50.6537065	36.7500326
4.86683305	5.46830182	67.591079	17.8215613
5.00248483	5.68608028	51.8807233	33.6013135
5.77297567	7.42147872	65.0113757	17.9064526
6.60299107	6.98678361	63.8741806	18.4646048
4.68920455	5.47824248	49.6368085	36.2251727
5.69376295	8.80727029	61.3442008	19.3274519
5.45614302	6.34015665	62.3197192	20.7444687
0.82055714	2.27118858	49.9028184	45.5484485
1.35078069	1.80834779	49.1880312	46.0863921
		0	0
0.85703246	2.5669796	50.886326	43.6810728
0.95973963	2.40977232	42.7656873	51.5575528
0.80043528	2.52754426	44.2698738	49.7956895
2.06580647	3.64114088	39.0754965	52.363559
1.36597878	2.72579271	39.3752907	54.2845384
1.27014188	4.19641182	35.9413799	56.6705855
2.06882376	3.42773224	39.0009499	52.1633924
1.86594462	4.2999686	43.0707849	47.3392999
3.74480373	4.75576024	62.9718611	24.7108612

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.