



Metabolic footprint analysis of metabolites that discriminate single and mixed yeast cultures at two key time-points during mixed culture alcoholic fermentations

Chuantao Peng¹ · Tiago Viana^{1,2} · Mikael Agerlin Petersen¹ · Flemming Hofmann Larsen¹ · Nils Arneborg¹ 

Received: 28 March 2018 / Accepted: 23 June 2018 / Published online: 4 July 2018
© Springer Science+Business Media, LLC, part of Springer Nature 2018

Abstract

Introduction There has been a growing interest towards creating defined mixed starter cultures for alcoholic fermentations. Previously, metabolite differences between single and mixed cultures have been explored at the endpoint of fermentations rather than during fermentations.

Objectives To create metabolic footprints of metabolites that discriminate single and mixed yeast cultures at two key time-points during mixed culture alcoholic fermentations.

Methods ¹H NMR- and GC–MS-based metabolomics was used to identify metabolites that discriminate single and mixed cultures of *Lachancea thermotolerans* (LT) and *Saccharomyces cerevisiae* (SC) during alcoholic fermentations.

Results Twenty-two metabolites were found when comparing single LT and mixed cultures, including both non-volatiles (carbohydrate, amino acid and acids) and volatiles (higher alcohols, esters, ketones and aldehydes). Fifteen of these compounds were discriminatory only at the death phase initiation (T1) and fifteen were discriminatory only at the death phase termination (T2) of LT in mixed cultures. Eight metabolites were discriminatory at both T1 and T2. These results indicate that specific metabolic changes may be descriptive of different LT growth behaviors. Fifteen discriminatory metabolites were found when comparing single SC and mixed cultures. These metabolites were all volatiles, and twelve metabolites were discriminatory only at T2, indicating that LT-induced changes in volatiles occur during the death phase of LT in mixed cultures and not during their initial growth stage.

Conclusions This work provides a detailed insight into yeast metabolites that differ between single and mixed cultures, and these data may be used for understanding and eventually predicting yeast metabolic changes in wine fermentations.

Keywords Single and mixed cultures · Yeast growth behaviors · Metabolic footprints · Metabolomics · Alcoholic fermentations

1 Introduction

A growing number of studies suggest that non-*Saccharomyces* species may contribute positively to the final taste and flavor of wine during mixed or sequential culture

fermentation with *Saccharomyces cerevisiae*. Thus, there has been a growing interest towards creating defined mixed or sequential starter cultures for alcoholic fermentations in which *S. cerevisiae* at some point grows together with the non-*Saccharomyces* species. In these kinds of fermentations, the non-*Saccharomyces* yeasts will grow together with *S. cerevisiae* during the first 1–2 days after inoculation with *S. cerevisiae*, but as the fermentation progresses the non-*Saccharomyces* species will typically die off leaving *S. cerevisiae* to dominate and complete the fermentation (Ciani et al. 2010; Liu et al. 2017; Wang et al. 2017; Comitini et al. 2011; Alonso-del-Real et al. 2017).

Metabolomics is defined as the quantitative analysis of all metabolites of a given cell or biofluid and has developed following the advances in data analytical methods as well

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s11306-018-1391-3>) contains supplementary material, which is available to authorized users.

✉ Nils Arneborg
na@food.ku.dk

¹ Department of Food Science, University of Copenhagen, Rolighedsvej 26, 1958 Frederiksberg C, Denmark

² Chr.Hansen A/S, Bøge Allé 10-12, 2970 Hørsholm, Denmark

as in MS and NMR instrumentation and sample preparation (Nicholson and Lindon 2008). GC–MS is a very high sensitivity (typically $\mu\text{g kg}^{-1}$ level) metabolomics method, but largely limited to volatile compounds (Kim et al. 2011; Ward et al. 2003). Liquid-state ^1H NMR can also be used for metabolomics studies and can simultaneously detect a range of proton-bearing compounds in a sample such as carbohydrates, amino acids, organic and fatty acids, amines, esters, ethers and lipids (Ward et al. 2003). Compared with MS, however, liquid-state ^1H NMR spectroscopy is characterized by relatively low sensitivity (Kim et al. 2011). In the context of alcoholic fermentations, GC–MS is often used to detect and analyze volatile compounds (Peng et al. 2013; Renault et al. 2015; Sadoudi et al. 2012; Tredoux et al. 2008) and ^1H NMR is applied for analysis of the non-volatiles (Nilsson et al. 2004; López-Rituerto et al. 2009, 2012, 2013; Son et al. 2009).

By metabolomics a metabolic footprint can be obtained. This is defined as an “imprint” of the extracellular metabolites of interest (Mapelli et al. 2008). Metabolic footprint analysis is typically performed during different growth phases of a microbial community, so metabolites of interest can be associated with any physiological changes in the microbial population (Mapelli et al. 2008).

Previous metabolomics studies of wine fermentations have shown that the metabolite compositions and/or concentrations in mixed yeast cultures may differ from those in their respective single cultures (Renault et al. 2015; Sadoudi et al. 2012; Howell et al. 2006). Those studies, however, focused on the metabolic differences at the endpoint of fermentation rather than during the fermentation. That is, these metabolic differences are the outcomes of the fermentations, and they do not necessarily reflect those occurring during the fermentations. Thus, there seems to be a lack of knowledge as to when specific yeasts contribute with specific metabolites during mixed culture fermentations. That kind of knowledge would provide valuable physiological insight into the dynamics of yeast interactions.

In this work, ^1H NMR- and GC–MS-based metabolomics was used for metabolic footprint analysis and identification of metabolites enabling discrimination between single and mixed culture metabolites at two key time-points of mixed culture alcoholic fermentations; that is, the time points where the death phase of the non-*Saccharomyces* yeast (*Lachancea thermotolerans*) was initiated (T1) and terminated (T2) in mixed cultures with *S. cerevisiae*.

2 Materials and methods

2.1 Yeast strains, growth media, and inoculation cultures

Saccharomyces cerevisiae BY4741 (Euroscarf, Germany) (SC) and *Lachancea thermotolerans* CBS6340 (Centraalbureau voor Schimmelcultures, The Netherlands) (LT) were maintained at pH 5.6 and 4 °C on Yeast Peptone Dextrose medium (YPG), containing 5 g/L yeast extract, 10 g/L peptone and 10 g/L glucose. To mimic natural grape must and winemaking practices, and ensure reproducibility of media composition, Synthetic Grape Must (SGM) (Viana et al. 2014) with defined composition was used to inoculate cultures and for the fermentation experiments. Briefly, it contains glucose (125 g/L), fructose (125 g/L), 19 amino acids, 3 weak organic acids (malic acid, citric acid and tartaric acid), 5 vitamins and different inorganic salts. Also, SO_2 was added (final concentration of 50 ppm), and the pH was adjusted to 3.3. It should be noted that *S. cerevisiae* BY4741 is an auxotrophic haploid strain derived from *S. cerevisiae* S288C, which is a well-characterized laboratory strain that was shown to have similar growth and fermentative profiles as an enological strain of *S. cerevisiae* in SGM fermentations (Viana et al. 2014). It may, however, have different metabolite production properties than those of industrial wine yeasts due to e.g. different ploidy of the two types of yeast. This issue needs further investigation. The inoculation of cultures was prepared by transferring 1 mL cells from YPG medium to glass tubes containing 10 mL SGM and incubation with agitation (140 rpm) at 25 °C for 24 h. Subsequently, these cells were transferred to 60 mL SGM in 100 mL shake flasks to an initial concentration of 2×10^6 cells/mL and incubated with agitation (140 rpm) at 25 °C for 48 h.

2.2 Single and mixed culture fermentations of *S. cerevisiae* and *L. thermotolerans*

Single and mixed culture fermentations of SC and LT were performed in 1 L blueCap bottles containing 0.8 L of SGM. In the mixed cultures, the initial viable cell density of SC and LT was 6×10^5 and 1.5×10^5 CFU/mL, respectively, resulting in a ratio between SC and LT of 4:1. In single cultures, the initial viable cell density of SC and LT was 1.2×10^6 and 3×10^5 CFU/mL, respectively. Fermentations were carried out under microaerobic conditions (with cotton caps) at 25 °C, without agitation, and all experiments were performed in duplicate.

Samples were withdrawn daily and diluted appropriately and SC and LT were enumerated using Wallerstein laboratory nutrient (WLN) agar (Oxoid). WLN plates were

incubated at 25 °C for 5 days before counting. Ethanol, glucose and fructose were measured daily by high-performance liquid chromatography (HPLC; HP series 1100, Hewlett-Packard Company, USA) as described by Kem-sawasd et al. (2015).

Cell-free supernatants from single and mixed cultures were sampled at two key time-points and stored at -20 °C for subsequent analysis by ¹H NMR and GC-MS. A simplified scheme of the experimental approach is shown in Fig. 1a.

2.3 ¹H NMR spectroscopy analysis and processing

Cell-free supernatants from single and mixed fermentations, harvested at key time-points as indicated, were defrosted, and the pH was measured and adjusted to 3.5 by the drop-wise addition of an aqueous solution of 1 M NaOH. Cell-free supernatants (540 μL) were transferred into a 5 mm

NMR tube together with 60 μL D₂O (containing 5.8 mM of the sodium salt of (trimethylsilyl) propanoic-2,2,3,3-d₄ acid (TSP-d₄)). ¹H NMR spectra were recorded on a Bruker Avance III 600 spectrometer operating at a Larmor frequency of 600.13 MHz for ¹H and equipped with a double tuned cryoprobe (TCI) for 5 mm (o.d.) sample tubes. The ¹H NMR spectra were recorded using the noesygppr1d pulse sequence to obtain an efficient suppression of the water resonance. All spectra were recorded using a recycle delay of 4 s, a spectral width of 30.04 ppm, an acquisition time of 2.73 s, 128 scans, 2 dummy scans and a receiver gain of 4.5. Subsequently the FID's were zero filled to 128 K data points, apodized by a Lorentzian linebroadening of 1 Hz and Fourier transformed. The spectra were hereafter phase and baseline corrected, and chemical shifts were referenced to the TSP-d₄ resonance at 0.0 ppm. Metabolites were assigned based on Chenomx NMR suite 8.1 (Chenomx Inc. Edmonton, Canada) and literature, and the MSI levels of metabolites were

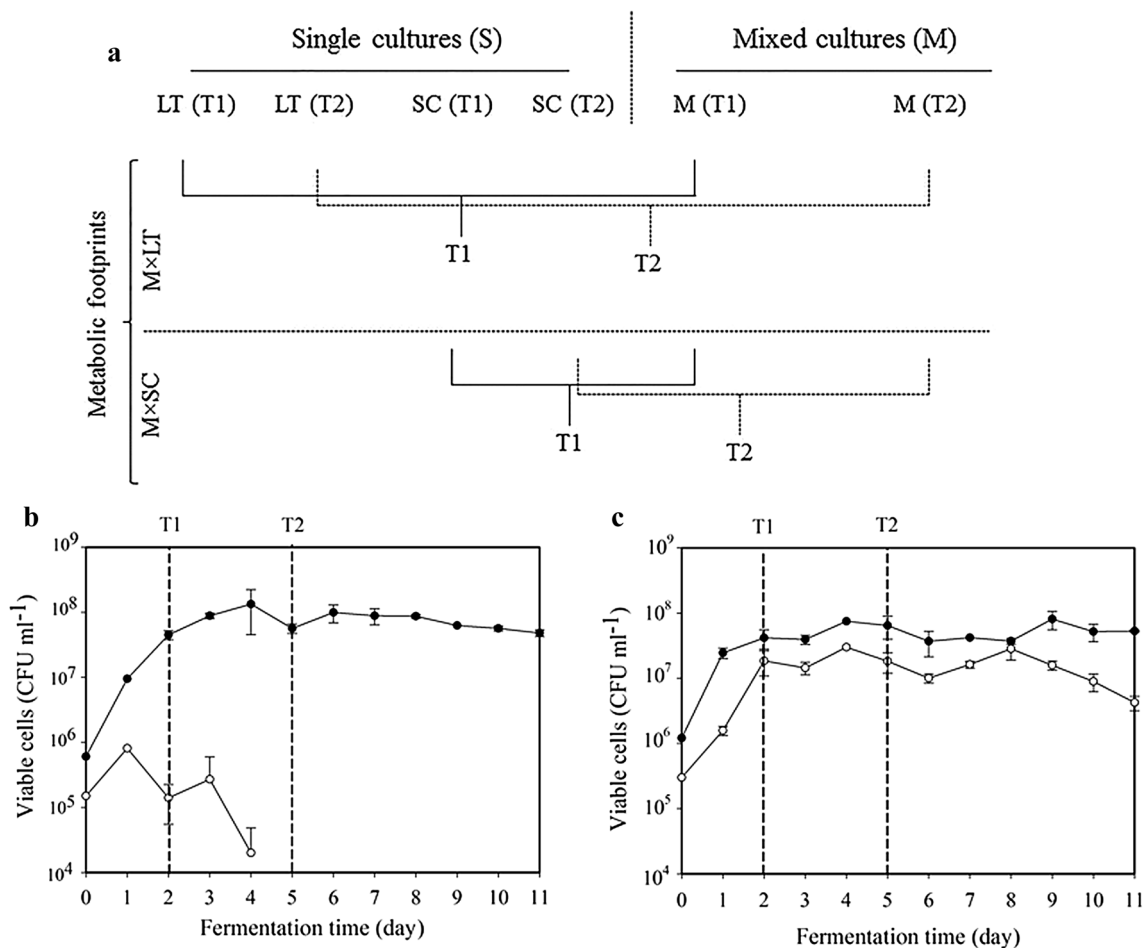


Fig. 1 Simplified scheme (a) of the experimental approach for metabolic footprint analysis of the differences between single (S) and mixed (M) cultures of *L. thermotolerans* (LT) and *S. cerevisiae* (SC) at two key time-points (T1 and T2) during mixed culture alcoholic

fermentation. Cell concentrations of LT (○) and SC (●) in mixed (b) and single (c) cultures. Data presented are mean values of two independent experiments ± standard deviation (error bars)

specified according to the Metabolomics Standards Initiative (MSI) (Sumner et al. 2007).

To compensate for chemical shift differences due to differences in pH or ionic strength amongst the samples, the *icoshift* program enabling alignment of resonances was employed (Savorani et al. 2010).

2.4 Dynamic headspace sampling (DHS)

Volatile compounds were collected in a dynamic headspace sampling (DHS) system. For each analysis, 20 mL sample was placed in a 100-mL gas washing flask equipped with a purge head, and 1.00 mL internal standard (5 ppm 4-methyl-1-pentanol) was added. A trap containing Tenax-TA (250 mg, mesh size 60/80, Buchem BV, Apeldoorn, The Netherlands) was attached to the purge head. The flask containing the sample was immersed in a laboratory water bath and held at 37 °C. Under magnetic stirring (200 rpm), the sample was then purged with nitrogen (100 mL/min) for 20 min to collect the volatiles. The traps were dry purged with nitrogen (100 mL/min) for 15 min to remove excess water trapped during purging. Sealed Tenax-TA traps with caps were kept at 5 °C before GC–MS analysis. The dynamic headspace collection was carried out in duplicate for all samples.

2.5 Gas chromatography–mass spectrometry (GC–MS)

Volatile compounds were analyzed using Gas chromatography–mass spectrometry (GC–MS) as described by Liu et al. (2015). Volatile compounds were identified by probability based matching of their mass spectra with those of a commercial database (Wiley275.L, HP product no. G1035A) and the minimum matching factor was set at 70%. Volatile compound identification was confirmed by comparison with retention indices (RI) of authentic reference compounds or retention indices reported in the literature. The MSI levels of volatiles were specified according to the Metabolomics Standards Initiative (MSI) (Sumner et al. 2007). All identified compounds were semi-quantified as peak areas in the total ion chromatogram (TIC).

2.6 Multivariate statistical analysis

The ^1H NMR spectra from the previous preprocessing steps were imported to Matlab R2016b (The Mathworks Inc. Natick, MA, USA). GC–MS data processing was carried out using MSD Chemstation G1701EA software (Version E.01.00.237, Agilent Technologies), and values were normalized by dividing peak areas of volatiles by that of internal standard from the same sample and were given as means based on independent duplicates. Interval partial least

squares discriminant analysis (*i*PLS–DA) (Kristensen et al. 2010; Nørgaard et al. 2000), principal component analysis (PCA) (Vold 1987), and student's *t* test were combined and used for variable selections from the acquired ^1H NMR and GC–MS data (see below). *i*PLS–DA was performed using the PLS toolbox in Matlab, PCA using LatentX 2.1 (Latent5, Copenhagen, Denmark) and *t* test using SPSS statistical package version 25.0 (SPSS Inc. Chicago, IL, USA).

For analysis of the ^1H NMR data, *i*PLS–DA was used for variable selections with respect to discrimination between samples from single or mixed culture fermentations and prior to *i*PLS–DA the ^1H NMR data were mean centered. Firstly, reference values of 0 and 1 for the PLS procedure were selected for the two different groups of fermentation. Then ^1H NMR spectra (0.0–11.4 ppm) were analyzed by *i*PLS–DA based on 100 intervals and the *i*PLS–DA procedure was performed with maximum number of ten latent variables. Hereafter intervals with a root mean square error of cross validation (RMSECV) lower than the full spectrum were selected. PCA and visual inspection of selected interval spectra were used for verifications. Intervals of interest were targeted as discriminatory metabolites, where PCA score plots of the mean-centered putative intervals, as well as visual inspections of the ^1H NMR spectra of putative intervals, showed clear differences between single and mixed cultures. For GC–MS data, *i*PLS–DA and *t* test were combined and used for variable selections. Prior to *i*PLS–DA the GC–MS data were autoscaled. The maximum number of latent variables was three. Then, every variable selected by *i*PLS–DA was verified again by *t* test of the given variable. Only the variables that had both low RMSECV and *p* value < 0.05 of *t* tests were determined as discriminatory metabolites.

3 Results

3.1 Identifying key time-points of mixed culture alcoholic fermentations

During the first day of both single LT and mixed cultures, LT grew at similar rates with a fivefold increase in the two cultures (Fig. 1b, c). Also, SC grew at similar rates both in single SC and mixed cultures during the first day (Fig. 1b, c). After the first day, the viable cell density of LT in single LT culture increased to between 1 and 3×10^7 CFU/mL and kept that value till day 10 (Fig. 1c). Conversely, in the mixed culture the LT cells began to die off after the first day, reaching a level below the detection limit at day 5 (Fig. 1b). However, in both single SC and mixed cultures, the cell viability of SC remained almost unchanged after day 1 at a level in the range of $1\text{--}10 \times 10^7$ CFU/mL (Fig. 1b, c).

Based on these observations, we defined the first key time-point (T1) of mixed culture alcoholic fermentations as

day 2 and the second key time-point (T2) as day 5. Sugar consumption and ethanol production rates were similar for the single SC and mixed cultures but slower in the single LT culture (Fig. S1).

3.2 Metabolic footprints of differences between the single LT and mixed cultures

The ^1H NMR spectra of single LT and mixed cultures at T1 and T2 are shown in Fig. S2. By comparison of the ^1H NMR spectra from the mixed cultures to those of the single LT cultures using *i*PLS-DA (Fig. 2a, b) and subsequent PCA, 9 discriminatory resonances were identified at T1 (0.88(d), 1.13(d), 1.27(t), 1.38(d), 2.07(s), 2.21(s), 2.23(d), 9.67(q), and 5.17(d) ppm, Fig. S3). These originate from the following 8 metabolites: isopentanol, 2,3-butanediol, ethyl acetate, lactic acid, acetic acid, acetoacetic acid, acetaldehyde and D-trehalose (Table S1 and Fig. 2c). At T2, the following 4

discriminatory metabolites were identified: 2,3-butanediol, lactic acid, acetic acid and 3-methyl-histidine (Table S1, Figs. 2c and S4). Three common discriminatory metabolites were found at T1 and T2, namely 2,3-butanediol, lactic acid and acetic acid (Fig. 2c). Thus, the main part of the metabolites discriminating the single LT and mixed cultures was observed at T1.

GC-MS analysis of single and mixed yeast cultures revealed a total of 50 volatiles that could be identified and quantified (Table S2). It should, however, be noted that there was a big difference in ethanol concentrations between single LT and mixed cultures; i.e. the ethanol concentrations at T2 reached 45 and 65 g/L in single LT and mixed cultures, respectively (Fig. S1). It is well-known that ethanol affects the extraction efficiency of volatiles during analysis (Cacho et al. 2013; Zhou et al. 2015). Through GC-MS and the multivariate statistical analysis described above, we found that, among the 50 volatiles, the concentrations

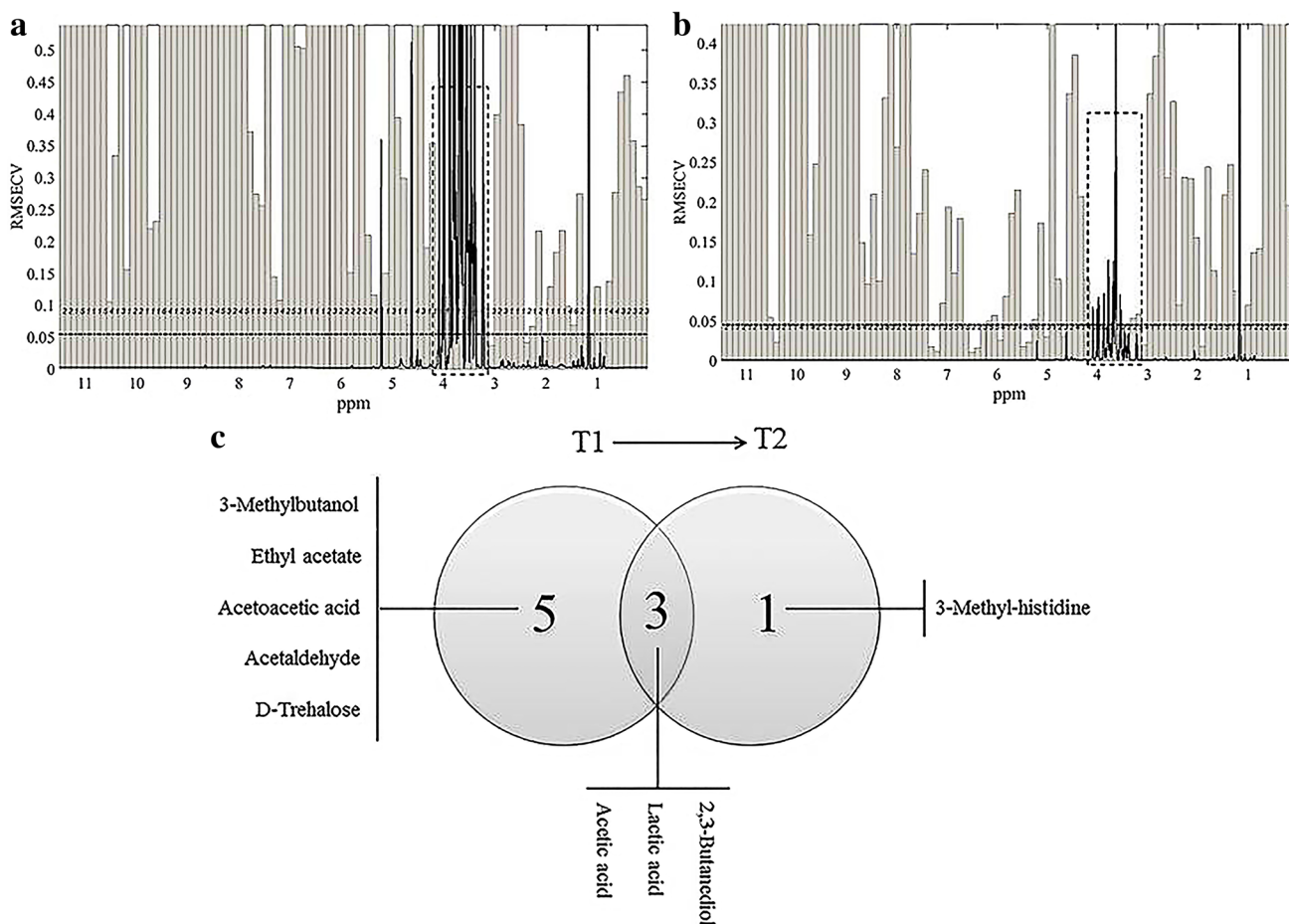


Fig. 2 *i*PLS-DA plot of ^1H NMR spectra discriminating between single LT and mixed cultures at T1 (a) and T2 (b) and corresponding discriminatory metabolites shown in Venn diagram (c). The carbohydrate region is surrounded by the dashed black boxes and the RMSECV of the global model is indicated by the dashed black horizontal line.

For further details on identifying intervals of interest, see Sect. 2. ^1H NMR spectra are those of two independent growth experiments. The overlap of Venn diagram (c) represents discriminatory metabolites between single LT and mixed cultures common to both T1 and T2

of 4-methyl-2-heptanone, ethyl pyruvate and acetoin were significantly affected (p value < 0.05) by different concentrations of ethanol (45 and 75 g/L), but those of the 47 other volatiles were only affected to a small extent (p value > 0.05) (data not shown). Thus, when comparing single LT and mixed cultures, 4-methyl-2-heptanone, ethyl pyruvate and acetoin were not considered.

Forty seven volatiles from the mixed cultures were compared to those of the single LT cultures by using *i*PLS-DA and *t* test (Fig. 3a, b). Eight discriminatory volatiles were identified at T1: ethyl acetate, 2-butanone, ethyl propionate, ethyl butanoate, 2-heptanone, ethyl octanoate, 2-methyl-tetrahydrothiophen-3-one and 2-phenethyl acetate (Fig. 3c).

At T2, 11 discriminatory volatiles were identified: ethyl propionate, ethyl butanoate, hexanal, 3-methyl-3-buten-1-ol, 2-heptanone, ethyl lactate, ethyl decanoate, 2-methyl-tetrahydrothiophen-3-one, octanol, ethyl 9-decenoate and 2-phenethyl acetate (Fig. 3c). Five discriminatory volatiles were found at both T1 and T2: ethyl propionate, ethyl butanoate, 2-heptanone, 2-methyl-tetrahydrothiophen-3-one and 2-phenethyl acetate (Fig. 3c).

Metabolic footprints of metabolites discriminating single LT and mixed cultures at T1 and T2 were constructed by combining the results from ^1H NMR and GC-MS (Fig. 5). At T1 the levels of D-trehalose, acetic acid, acetoacetic acid, 2,3-butanediol, 3-methylbutanol,

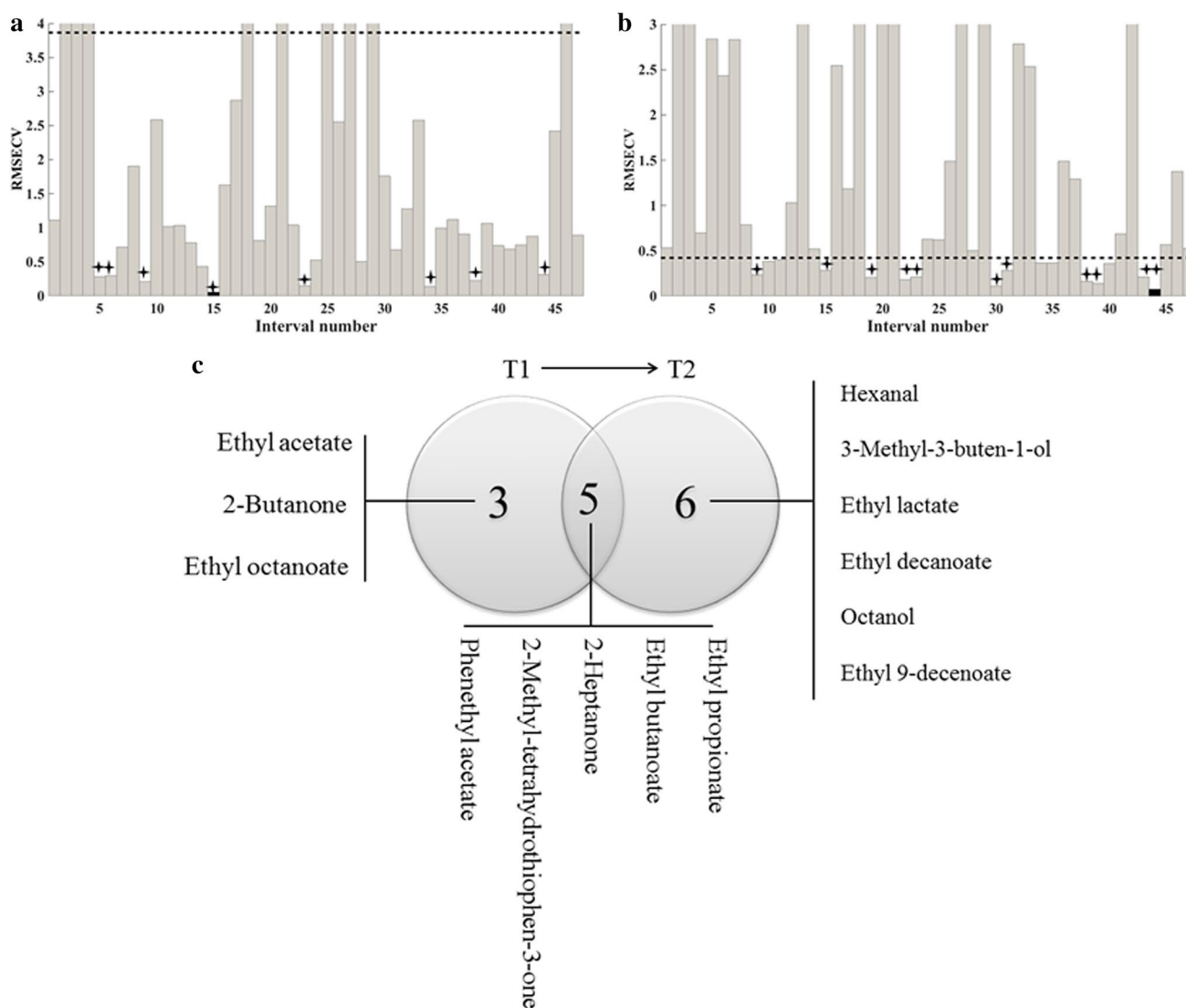


Fig. 3 *i*PLS-DA plot of GC-MS data discriminating between single LT and mixed cultures at T1 (a) and T2 (b) and corresponding discriminatory metabolites shown in Venn diagram (c). GC-MS data are mean values of two independent growth experiments and were analyzed by *i*PLS-DA based on 47 intervals. The RMSECV of the global

model was indicated by the dashed black horizontal line. For further details on identifying volatiles of interest, see Sect. 2. Black cross symbols denote significant difference ($p < 0.05$). The overlap of Venn diagram (c) represents discriminatory metabolites between single LT and mixed cultures common to both T1 and T2

ethyl acetate, ethyl octanoate, ethyl butanoate, 2-phenethyl acetate, 2-heptanone, 2-methyl-tetrahydrothiophen-3-one, and acetaldehyde were higher, and the levels of lactic acid, ethyl propionate and 2-butanone were lower in mixed cultures than in the single LT cultures (Fig. 5).

At T2 the levels of 3-methyl-histidine, acetic acid, 2,3-butanediol, octanol, ethyl butanoate, ethyl decanoate, ethyl 9-decenoate, 2-phenethyl acetate, 2-heptanone and 2-methyl-tetrahydrothiophen-3-one were higher, and the levels of lactic acid, 3-methyl-3-buten-1-ol, ethyl propionate, ethyl lactate, and hexanal were lower in mixed cultures than in the single LT cultures (Fig. 5).

At both time points the levels of acetic acid, 2,3-butanediol, ethyl butanoate, 2-phenethyl acetate, 2-heptanone, and 2-methyl-tetrahydrothiophen-3-one were higher, and the levels of lactic acid and ethyl propionate were lower in mixed cultures than in the single LT cultures (Fig. 5).

3.3 Metabolic footprints of differences between the single SC and mixed cultures

The ^1H NMR spectra of single SC and mixed cultures at T1 and T2 are shown in Fig. S2. Even though a couple of spectral intervals were identified by *i*PLS-DA, the subsequent PCA and visual inspection of the selected intervals did not provide a clear difference between single SC and mixed cultures (Figs. S5 and S6). These results indicate that the NMR detectable metabolites in the mixed cultures were not changed by the presence of LT in comparison to single SC cultures.

Fifty volatiles from the mixed cultures were compared to those of the single SC cultures by using *i*PLS-DA and *t* test (Fig. 4a, b). At T1, 3 volatiles provided discrimination between single SC and mixed cultures. These were 3-methylbutanal, ethyl 9-decenoate and ethyl decanoate (Fig. 4c). At T2, 14 discriminatory volatiles were

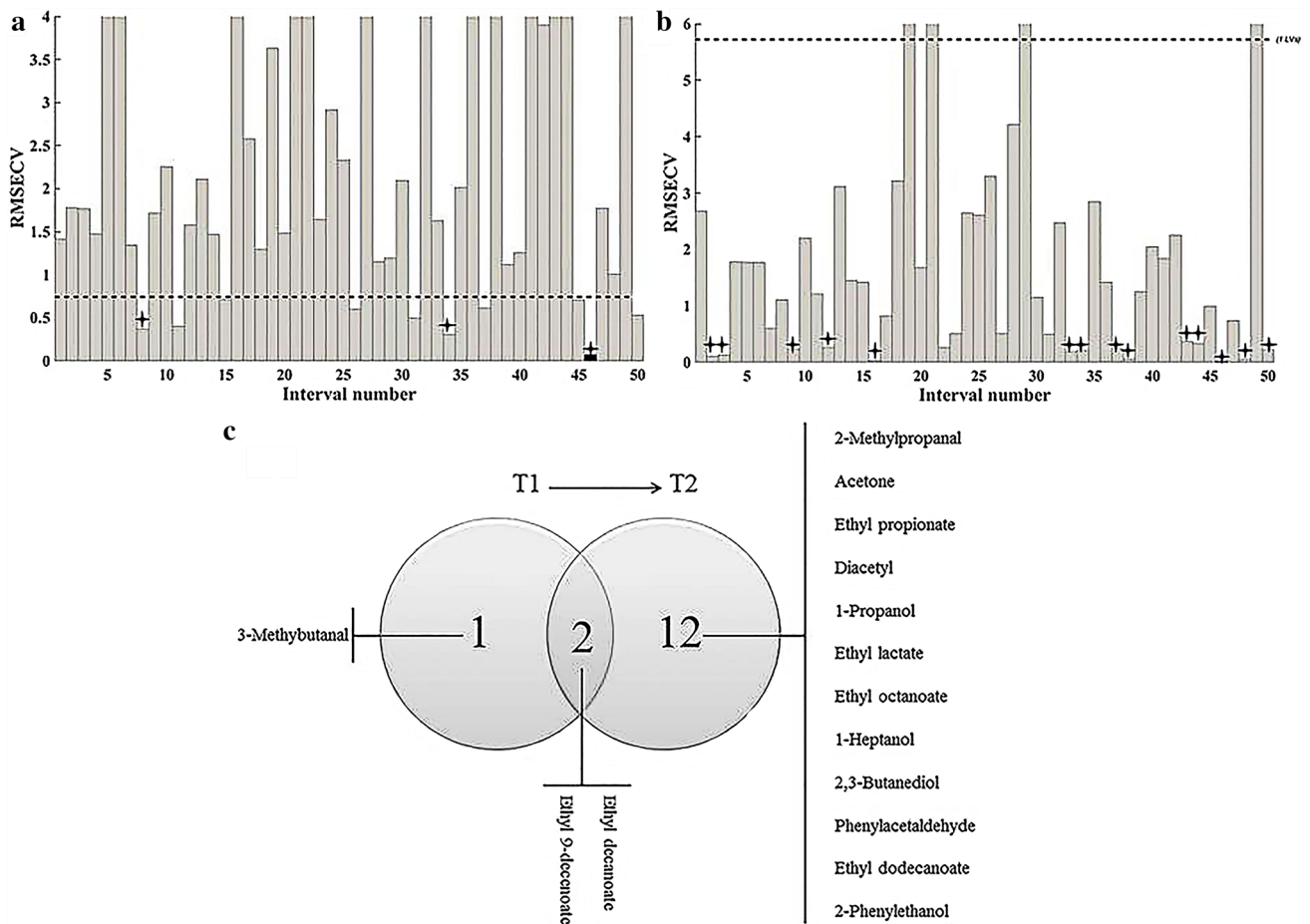
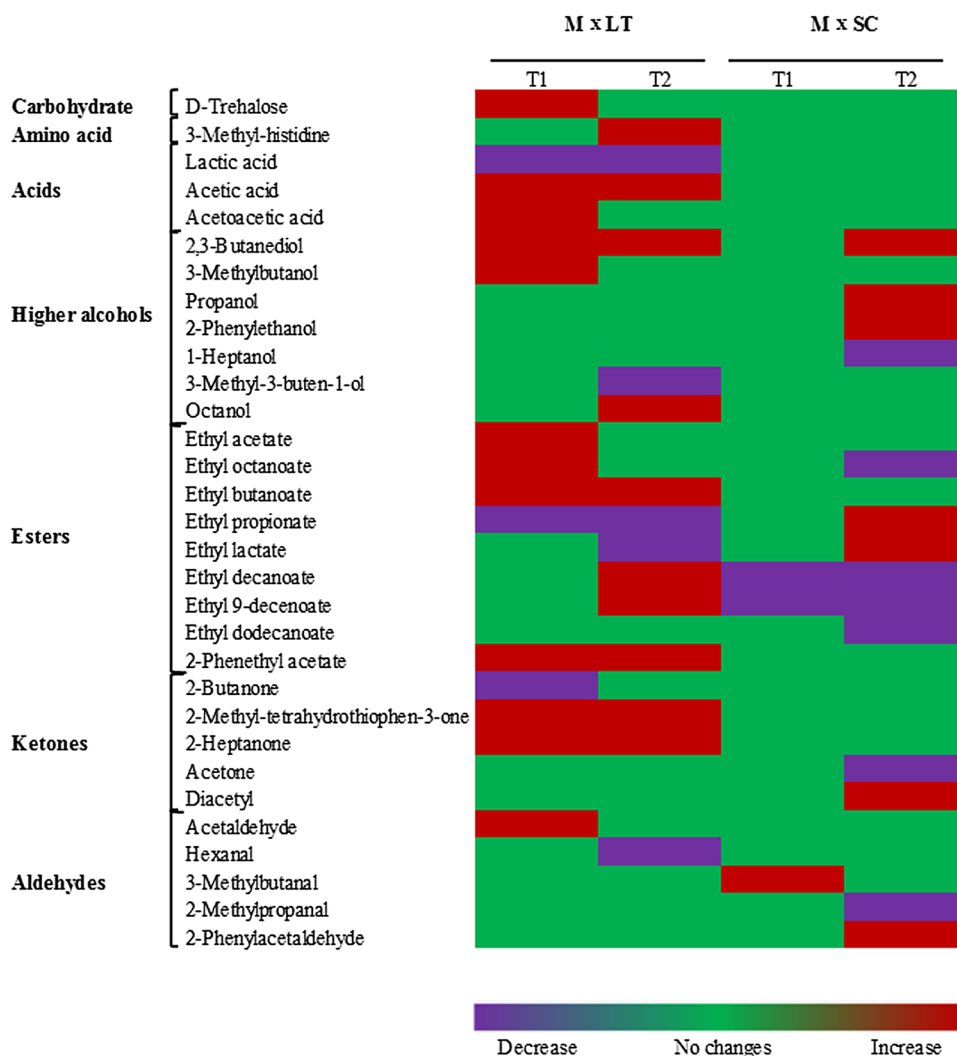


Fig. 4 *i*PLS-DA plot of GC-MS data discriminating between single SC and mixed cultures at T1 (a) and T2 (b) and corresponding discriminatory metabolites shown in Venn diagram (c). GC-MS data are mean values of two independent growth experiments and were analyzed by *i*PLS-DA based on 50 intervals. The RMSECV of the global

model was indicated by the dashed black horizontal line. For further details on identifying volatiles of interest, see Sect. 2. Black cross symbols denote significant difference ($p < 0.05$). The overlap of Venn diagram (c) represents discriminatory metabolites between single SC and mixed cultures common to both T1 and T2

Fig. 5 Metabolic footprints of metabolites discriminating single and mixed cultures of *L. thermotolerans* (LT) and *S. cerevisiae* (SC) at T1 and T2, represented as increased (red), unchanged (green), and decreased (purple) levels in mixed cultures (M) as compared to those of single cultures



identified as 2-methylpropanal, acetone, ethyl propionate, ethyl decanoate, diacetyl, 1-propanol, ethyl lactate, ethyl octanoate, 1-heptanol, 2,3-butanediol, phenylacetaldehyde, ethyl 9-decenoate, ethyl dodecanoate and 2-phenylethanol (Fig. 4c). Two volatiles enabling discrimination were found at both T1 and T2, namely ethyl decanoate and ethyl 9-decenoate (Fig. 4c). Thus, the volatile composition in the mixed cultures differed more at T2 than at T1, as compared with the single SC cultures.

Based on the results mentioned above, metabolic footprints of the metabolites discriminating single SC and mixed cultures at T1 and T2 were established (Fig. 5). At T1, the level of 3-methylbutanal was higher in the mixed cultures than in the single SC cultures, and the levels of ethyl decanoate and ethyl 9-decenoate were lower (Fig. 5). At T2, the levels of 2,3-butanediol, 1-propanol, 2-phenylethanol, ethyl lactate, ethyl propionate, diacetyl and 2-phenylacetaldehyde were higher in the mixed cultures than in the single SC cultures, and the levels of 1-heptanol, ethyl octanoate,

ethyl decanoate, ethyl laurate, acetone, 2-methylpropanal and ethyl 9-decenoate were lower (Fig. 5). At both T1 and T2, the levels of ethyl decanoate and ethyl 9-decenoate were lower in the mixed cultures than in the single SC cultures (Fig. 5).

4 Discussion

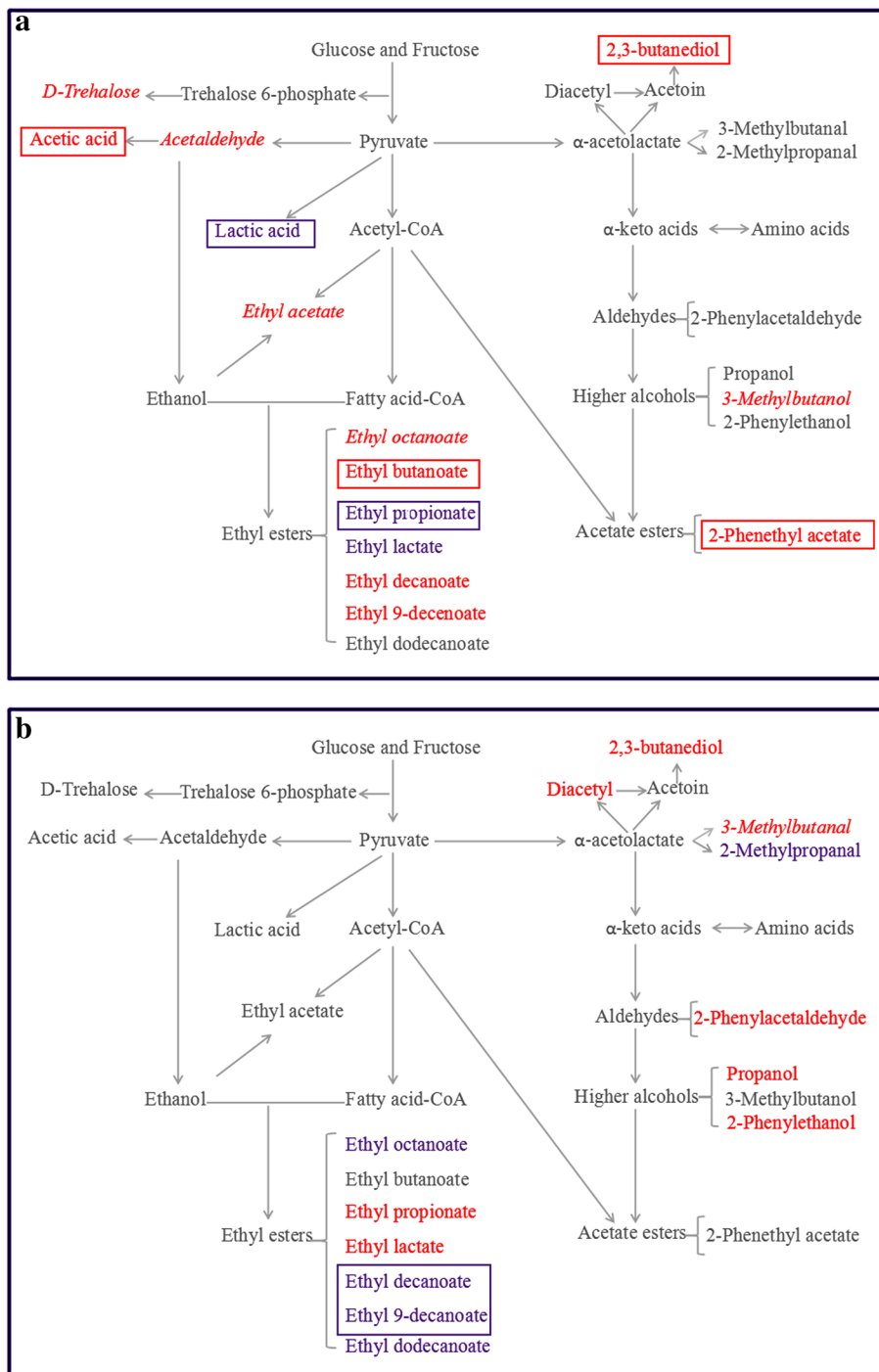
To the best of our knowledge, this study is the first of its kind investigating metabolic differences between single and mixed cultures during alcoholic fermentations using both ^1H NMR- and GC-MS-based metabolomics. ^1H liquid-state NMR is used to analyze the liquid part of the samples, whereas GC-MS is applied for analysis of the volatiles. Besides analyzing different parts of the samples the two analytical methods differ in their sensitivity (Kim et al. 2011; Ward et al. 2003). The detection limit for GC-MS is often in the order of ppb (parts per billion), whereas compounds

with a concentration below 0.1 mM are difficult to observe by ¹H NMR. Combined the two methodologies provide complementary information about a range of compounds of the samples at specific stages of fermentation.

Known metabolic pathways of the different discriminatory metabolites at T1 and T2 found in this study can be seen in Fig. 6. Most esters in yeast are formed by a condensation reaction between acetyl/fatty acid-CoA and an alcohol, and the use of acetyl-CoA or fatty acid-CoA

divides esters into two different categories, acetate esters and fatty acid ethyl esters, respectively (Dzialo et al. 2017). Interestingly, among the volatiles providing discrimination between single and mixed cultures at either T1 or T2, five compounds belong to the ethyl esters: ethyl octanoate, ethyl propionate, ethyl lactate, ethyl decanoate, and ethyl 9-decanoate. The physiological significance of this observation, however, still needs to be elucidated.

Fig. 6 Pathways of metabolites that increased (red), remained unchanged (grey), or decreased (purple) in mixed cultures as compared to those of single LT cultures (a) and single SC cultures (b) at T1 (italic), T2 (upright), or both at T1 and T2 (box)



Higher alcohols are formed by decarboxylation and subsequent reduction of α -keto acids produced as intermediates of amino acid catabolism; i.e. by the so-called Ehrlich pathway (Ugliano and Henschke 2009; Dzialo et al. 2017). Accordingly, amino acid degradation accounts for most of the higher alcohols formed during alcoholic fermentations (Ugliano and Henschke 2009). In our study, the amino acid precursors for the discriminatory branched chain aliphatic alcohols 3-methylbutanol (or isoamyl alcohol) and 1-propanol are leucine and threonine, respectively, and for the discriminatory aromatic alcohol 2-phenylethanol, it is phenylalanine (Ugliano and Henschke 2009; Dzialo et al. 2017). It should be noted that metabolic pathways of the discriminatory higher alcohols 1-heptanol, 3-methyl-3-buten-1-ol and octanol (Fig. 5) have not, as yet, been described in yeasts.

In yeasts, aldehydes and ketones are produced from sugar metabolism (Ugliano and Henschke 2009). Among the discriminatory ketones and aldehydes, the metabolic pathways of acetaldehyde, two branched-chain aldehydes (3-methylbutanal and 2-methylpropanal), diacetyl and 2-phenylacetaldehyde are described in yeasts (Ugliano and Henschke 2009; Dzialo et al. 2017); see Fig. 6. The metabolic pathways, however, of acetone, 2-butanone, 2-methyl-tetrahydrothiophen-3-one, 2-heptanone, and hexanal (Fig. 5) seem not, as yet, to have been described.

Previous metabolome studies from alcoholic fermentations using single and mixed yeast cultures typically report data from the end of fermentations (Renault et al. 2015; Sadoudi et al. 2012; Howell et al. 2006; Ciani et al. 2006). Thus, these studies report the metabolite differences between single and mixed cultures according to the final result of alcoholic fermentations. Also, these studies typically focus on analysis of volatiles by GC-MS. Here, we present both GC-MS- and ^1H NMR-data describing the metabolic differences between single and mixed cultures at two different stages during mixed culture alcoholic fermentations; i.e. at the beginning (T1) and the end (T2) of the death phase of the non-*Saccharomyces* yeast in mixed cultures. In order to check, whether data from these time-points actually differ from those at the end of fermentation, we made a metabolic footprint analysis of the metabolites discriminating the single and mixed cultures at day 10 similar to those at day 2 (T1) and day 5 (T2), the results of which are shown in Figs. S7–S9. The fact that different metabolites enabled discrimination between single and mixed cultures at day 10 (Figs. S7–S9) and day 5 (Figs. 2, 3, 4) suggests that the dominant yeast changes the metabolic profiles of mixed cultures from day 5 to day 10 after LT has died out.

We have found two compounds in alcoholic fermentations that have never been described within this context before: D-trehalose and 3-methyl-histidine. D-Trehalose is well-known for its role as a carbohydrate reserve and stress protectant in yeast, and it accumulates intracellularly during

adverse environmental conditions (Bandara et al. 2009; Ocón et al. 2007; Puig-Castellví et al. 2015). However, it has never been reported as part of the extracellular environment during alcoholic fermentations. Whether this phenomenon is due to trehalose being secreted out of intact yeast cells or to lysis of dead yeast cells during fermentation, or perhaps to a combination of both, remains to be investigated. In the yeast SC, histidine protein methylation is an unusual post-translational modification and the large ribosomal subunit protein Rp13p is methylated at histidine 243 (Al-Hadid et al. 2014). Histidine is available in the SGM and methylation may be induced at T2 due to a metabolic pathway that has not, as yet, been described.

Takahashi et al. (2008) reported that metabolic footprint analysis was a useful method to build growth-metabolite associations; that is, the relationship between growth and metabolites based on metabolomics approaches, and they used a metabolomics approach to analyze growth-specific metabolites of bacteria by constructing a linear relationship between OD600 values and metabolites profiles (Takahashi et al. 2008). Also, the relationship between growth and metabolites in plants has been explored (Lee et al. 2012). Metabolites of different-sized *Aloe vera* plants were determined using MS-based metabolomics approaches, and metabolite changes associated with growth and development were revealed (Lee et al. 2012). Here, we are the first to describe yeast growth-metabolite associations at two key time-points of mixed culture alcoholic fermentations.

Twenty-two metabolites provided discrimination between single LT and mixed cultures and comprised both non-volatiles (carbohydrate, amino acid and acids) and volatiles (higher alcohols, esters, ketones and aldehydes). Some metabolites were important at the LT death phase initiation (T1) and some at the death phase termination (T2). These results indicate that specific metabolic changes may be descriptive of different LT growth behaviors during mixed culture alcoholic fermentations.

The higher levels of 3-methyl-histidine, acetic acid, acetoacetic acid, 2,3-butanediol, 3-methylbutanol, octanol, ethyl butanoate, ethyl octanoate, ethyl acetate, ethyl decanoate, ethyl 9-decenoate, 2-phenethyl acetate, 2-methyl-tetrahydrothiophen-3-one, 2-heptanone and acetaldehyde in mixed cultures compared to single LT cultures, combined with the observed inhibition of LT in mixed cultures but not in single LT cultures, indicate that these compounds have inhibiting effects on the growth of LT. Further work is, however, required to better understand the roles of these compounds on the growth of LT during mixed culture alcoholic fermentations.

For the mixed cultures, levels of lactic acid, 3-methyl-3-buten-1-ol, ethyl propionate, ethyl lactate, 2-butanone and hexanal were lower than in single LT cultures. LT is a natural lactic acid producer (Zara et al. 2014), whereas SC

is not. As LT died in mixed cultures, it makes sense that the lactic acid concentration, and thereby also ethyl lactate that is synthesized from ethanol and lactic acid, were lower in mixed cultures than in single LT cultures. At T1, the levels of 2-butanone and ethyl propionate were lower than in any single cultures and the lower levels of these two metabolites may be the result of negative metabolic interactions. At T2, the levels of 3-methyl-3-buten-1-ol and hexanal were much lower than in single LT cultures and the lower levels of these two metabolites may be caused by the death of LT in mixed cultures. These phenomena, however, need to be further investigated.

When comparing the mixed cultures with the single SC cultures, a rather different growth-metabolite association was observed. The cell viability of SC remained almost unchanged in the two cultures, but volatiles (higher alcohols, esters, ketones and aldehydes) enabling discrimination between the single SC and mixed cultures were found as seen in Figs. 4 and 5, especially at T2. It is well known that non-*Saccharomyces* yeasts contribute to the flavor of wine, as described by a plethora of authors (Ciani et al. 2010; Renault et al. 2015; Sadoudi et al. 2012; Padilla et al. 2016; Varela et al. 2016). This knowledge is mainly based on analysis of the final wines. Our study indicates that the changes in volatiles primarily occur during the death phase of the non-*Saccharomyces* yeasts in mixed cultures and not during their initial growth stage.

5 Conclusions

In conclusion, this work provides a detailed insight into metabolites that discriminate single and mixed yeast cultures during alcoholic fermentations as determined by ¹H NMR- and GC-MS-metabolomics. Our results show that metabolites enabling discrimination between single LT and mixed cultures were highly dependent on the LT growth behaviors during mixed culture alcoholic fermentations. Furthermore, when comparing single SC and mixed cultures, the LT-induced changes in volatiles occurred during the death phase of LT in mixed cultures and not during their initial growth stage. These data may be used for understanding and eventually predicting yeast growth behaviors and metabolic changes during wine fermentations, where mixed yeast starter cultures may be used.

Acknowledgements The authors would like to thank Janne Margrethe Benjaminsen for excellent technical assistance.

Author contributions NA, CP and TV conceived and designed research. CP conducted experiments. MAP and FHL contributed new reagents and analytical tools. CP and FHL analyzed data. CP and NA wrote the manuscript. All authors read, revised and approved the manuscript.

Funding This study was funded by Faculty of Science, University of Copenhagen and Chinese Scholarship Council (201406300048).

Data availability The data that support the findings of this study are available within the article and its supplementary information files.

Compliance with ethical standards

Conflict of interest Chuantao Peng, Tiago Viana, Mikael Agerlin Petersen, Flemming Hofmann Larsen and Nils Arneborg declares that they have no conflict of interest.

Ethical approval This article does not contain any studies with human or animal subjects performed by any of the authors.

References

- Al-Hadid, Q., Roy, K., Munroe, W., Dzialo, M. C., Chanfreau, G. F., & Clarke, S. G. (2014). Histidine methylation of yeast ribosomal protein Rpl3p is required for proper 60S subunit assembly. *Molecular and Cellular Biology*, *34*, 2903–2916.
- Alonso-del-Real, J., Contreras-Ruiz, A., Castiglioni, G. L., Barrio, E., & Querol, A. (2017). The use of mixed populations of *Saccharomyces cerevisiae* and *S. kudriavzevii* to reduce ethanol content in wine: Limited aeration, inoculum proportions, and sequential inoculation. *Frontiers in Microbiology*, *8*, 2087.
- Bandara, A., Fraser, S., Chambers, P. J., & Stanley, G. A. (2009). Trehalose promotes the survival of *Saccharomyces cerevisiae* during lethal ethanol stress, but does not influence growth under sublethal ethanol stress. *FEMS Yeast Research*, *9*, 1208–1216.
- Cacho, J. I., Campillo, N., Viñas, P., & Hernández-Córdoba, M. (2013). Stir bar sorptive extraction with EG-silicone coating for bisphenols determination in personal care products by GC-MS. *Journal of Pharmaceutical and Biomedical Analysis*, *78–79*, 255–260.
- Ciani, M., Beco, L., & Comitini, F. (2006). Fermentation behaviour and metabolic interactions of multistarter wine yeast fermentations. *International Journal of Food Microbiology*, *108*, 239–245.
- Ciani, M., Comitini, F., Mannazzu, I., & Domizio, P. (2010). Controlled mixed culture fermentation: A new perspective on the use of non-*Saccharomyces* yeasts in winemaking. *FEMS Yeast Research*, *10*, 123–133.
- Comitini, F., Gobbi, M., Domizio, P., Romani, C., Lencioni, L., Mannazzu, I., et al. (2011). Selected non-*Saccharomyces* wine yeasts in controlled multistarter fermentations with *Saccharomyces cerevisiae*. *Food Microbiology*, *28*, 873–882.
- Dzialo, M. C., Park, R., Steensels, J., Lievens, B., & Verstrepen, K. J. (2017). Physiology, ecology and industrial applications of aroma formation in yeast. *FEMS Microbiology Reviews*, *41*, S95–S128.
- Howell, K. S., Cozzolino, D., Bartowsky, E. J., Fleet, G. H., & Henschke, P. A. (2006). Metabolic profiling as a tool for revealing *Saccharomyces* interactions during wine fermentation. *FEMS Yeast Reviews*, *6*, 91–101.
- Kemsawasd, V., Viana, T., Ardö, Y., & Arneborg, N. (2015). Influence of nitrogen sources on growth and fermentation performance of different wine yeast species during alcoholic fermentation. *Applied Microbiology and Biotechnology*, *99*, 10191–10207.
- Kim, H. K., Choi, Y. H., & Verpoorte, R. (2011). NMR-based plant metabolomics: Where do we stand, where do we go? *Trends in Biotechnology*, *29*, 267–275.
- Kristensen, M., Savorani, F., Ravn-Haren, G., Poulsen, M., Markowski, J., Larsen, F. H., et al. (2010). NMR and interval PLS as reliable

- methods for determination of cholesterol in rodent lipoprotein fractions. *Metabolomics*, 6, 129–136.
- Lee, S., Do, S., Kim, S. Y., Kim, J., Jin, Y., & Lee, C. H. (2012). Mass spectrometry-based metabolite profiling and antioxidant activity of *Aloe vera* (*Aloe barbadensis* Miller) in different growth stages. *Journal of Agricultural and Food Chemistry*, 60, 11222–11228.
- Liu, J., Arneborg, N., Toldam-Andersen, T. B., Zhang, S., Petersen, M. A., & Bredie, W. L. (2017). Impact of sequential co-culture fermentations on flavour characters of Solaris wines. *European Food Research and Technology*, 243, 437–445.
- Liu, J., Toldam-Andersen, T. B., Petersen, M. A., Zhang, S., Arneborg, N., & Bredie, W. L. P. (2015). Instrumental and sensory characterisation of Solaris white wines in Denmark. *Food Chemistry*, 166, 133–142.
- López-Rituerto, E., Avenzoza, A., Busto, J. H., & Peregrina, J. M. (2013). NMR study of histidine metabolism during alcoholic and malolactic fermentations of wine and their influence on histamine production. *Journal of Agricultural and Food Chemistry*, 61, 9464–9469.
- López-Rituerto, E., Cabredo, S., López, M., Avenzoza, A., Busto, J. H., & Peregrina, J. M. (2009). A thorough study on the use of quantitative ¹H NMR in Rioja red wine fermentation processes. *Journal of Agricultural and Food Chemistry*, 57, 2112–2118.
- López-Rituerto, E., Savorani, F., Avenzoza, A., Busto, J. H., Peregrina, J. M., & Engelsen, S. B. (2012). Investigations of La Rioja terroir for wine production using ¹H NMR metabolomics. *Journal of Agricultural and Food Chemistry*, 60, 3452–3461.
- Mapelli, V., Olsson, L., & Nielsen, J. (2008). Metabolic footprinting in microbiology: Methods and applications in functional genomics and biotechnology. *Trends in Biotechnology*, 26, 490–497.
- Nicholson, J. K., & Lindon, J. C. (2008). Systems biology: Metabonomics. *Nature*, 455, 1054–1056.
- Nilsson, M., Duarte, I. F., Almeida, C., Delgadillo, I., Goodfellow, B. J., Gil, A. M., et al. (2004). High-resolution NMR and diffusion-ordered spectroscopy of port wine. *Journal of Agricultural and Food Chemistry*, 52, 3736–3743.
- Nørgaard, L., Saudland, A., Wagner, J., Nielsen, J. P., Munck, L., & Engelsen, S. B. (2000). Interval partial least squares regression (iPLS): A comparative chemometric study with an example from the near infrared spectroscopy. *Applied Spectroscopy*, 54, 413–419.
- Ocón, A., Hampp, R., & Requena, N. (2007). Trehalose turnover during abiotic stress in arbuscular mycorrhizal fungi. *New Phytologist*, 174, 879–891.
- Padilla, B., Gil, J. V., & Manzanares, P. (2016). Past and future of non-*Saccharomyces* yeasts: From spoilage microorganisms to biotechnological tools for improving wine aroma complexity. *Frontiers in Microbiology*, 7, 411.
- Peng, C. T., Wen, Y., Tao, Y. S., & Lan, Y. Y. (2013). Modulating the formation of Meili wine aroma by prefermentative freezing process. *Journal of Agricultural and Food Chemistry*, 61, 1542–1553.
- Puig-Castellví, F., Alfonso, I., Piña, B., & Tauler, R. (2015). A quantitative ¹H NMR approach for evaluating the metabolic response of *Saccharomyces cerevisiae* to mild heat stress. *Metabolomics*, 11, 1612–1625.
- Renault, P., Coulon, J., de Revel, G., Barbe, J. C., & Bely, M. (2015). Increase of fruity aroma during mixed *T. delbrueckii*/*S. cerevisiae* wine fermentation is linked to specific esters enhancement. *International Journal of Food Microbiology*, 207, 40–48.
- Sadoudi, M., Tourdot-Maréchal, R., Rousseaux, S., Steyer, D., Gallardo-Chacón, J. J., Ballester, J., et al. (2012). Yeast-yeast interactions revealed by aromatic profile analysis of Sauvignon Blanc wine fermented by single or co-culture of non-*Saccharomyces* and *Saccharomyces* yeasts. *Food Microbiology*, 32, 243–253.
- Savorani, F., Tomasi, G., & Engelsen, S. B. (2010). icoshift: A versatile tool for the rapid alignment of 1D NMR spectra. *Journal of Magnetic Resonance*, 202, 190–202.
- Son, H. S., Hwang, G. S., Kim, K. M., Kim, E. Y., van den Berg, F., Park, W. M., et al. (2009). ¹H NMR-based metabolomic approach for understanding the fermentation behaviors of wine yeast strains. *Analytical Chemistry*, 81, 1137–1145.
- Sumner, L. W., Amberg, A., Barrett, D., Beale, M. H., Beger, R., Daykin, C. A., et al. (2007). Proposed minimum reporting standards for chemical analysis. *Metabolomics*, 3, 211–221.
- Takahashi, H., Kai, K., Shinbo, Y., Tanaka, K., Ohta, D., Oshima, T., et al. (2008). Metabolomics approach for determining growth-specific metabolites based on Fourier transform ion cyclotron resonance mass spectrometry. *Analytical and Bioanalytical Chemistry*, 391, 2769–2782.
- Tredoux, A., De Villiers, A., Májek, P., Lynen, F., Crouch, A., & Sandra, P. (2008). Stir bar sorptive extraction combined with GC-MS analysis and chemometric methods for the classification of South African wines according to the volatile composition. *Journal of Agricultural and Food Chemistry*, 56, 4286–4296.
- Ugliano, M., & Henschke, P. A. (2009). Yeasts and wine flavour. In M. V. Moreno-Arribas & M. C. Polo (Eds.), *Wine chemistry and biochemistry* (pp. 313–392). New York: Springer.
- Varela, C., Sengler, F., Solomon, M., & Curtin, C. (2016). Volatile flavour profile of reduced alcohol wines fermented with the non-conventional yeast species *Metschnikowia pulcherrima* and *Saccharomyces uvarum*. *Food Chemistry*, 209, 57–64.
- Viana, T., Loureiro-Dias, M. C., & Prista, C. (2014). Efficient fermentation of an improved synthetic grape must by enological and laboratory strains of *Saccharomyces cerevisiae*. *AMB Express*, 4, 16.
- Vold, S. (1987). Principal component analysis. *Chemometrics and Intelligent Laboratory Systems*, 2, 37–52.
- Wang, X. C., Li, A. H., Dizey, M., Ullah, N., Sun, W. X., & Tao, Y. S. (2017). Evaluation of aroma enhancement for “Ecolly” dry white wines by mixed inoculation of selected *Rhodotorula mucilaginosa* and *Saccharomyces cerevisiae*. *Food Chemistry*, 228, 550–559.
- Ward, J. L., Harris, C., Lewis, J., & Beale, M. H. (2003). Assessment of ¹H NMR spectroscopy and multivariate analysis as a technique for metabolite fingerprinting of *Arabidopsis thaliana*. *Phytochemistry*, 62, 949–957.
- Zara, G., Ciani, M., Domizio, P., Zara, S., Budroni, M., Carboni, A., et al. (2014). A culture-independent PCR-based method for the detection of *Lachancea thermotolerans* in wine. *Annals of Microbiology*, 64, 403–406.
- Zhou, Q., Qian, Y., & Qian, M. C. (2015). Analysis of volatile phenols in alcoholic beverage by ethylene glycol-polydimethylsiloxane based stir bar sorptive extraction and gas chromatography-mass spectrometry. *Journal of Chromatography A*, 1390, 22–27.