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Effects of sequential inoculation of *Lachancea thermotolerans* and *Oenococcus oeni* on chemical composition of spent coffee grounds hydrolysates

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ABSTRACT

Spent coffee grounds (SCG) disposal is an environmental problem. These residues from coffee brewing and instant coffee production have potential to produce novel alcoholic beverages. SCG valorization through sequential alcoholic and malolactic fermentation was investigated using a yeast, *Lachancea thermotolerans* Concerto and a lactic acid bacterium (LAB), *Oenococcus oeni* Lalvin 31 in this study. Our results showed that sequential inoculation prevented early death of yeast confronted when simultaneous inoculation was adopted, allowing for growth and persistence of both yeast and LAB till the end of fermentation. Adequate ethanol production (4.91 ± 0.13 %, v/v) with low residual sugar content was also attained. In addition, relatively lower levels of acetic, lactic, and succinic acids were produced by sequential inoculation than that of simultaneous inoculation. Furthermore, SCG hydrolysates fermented via sequential inoculation had the widest variety of volatiles (e.g. esters and ketones). Overall, our results indicated that sequential inoculation of *L. thermotolerans* and *O. oeni* in SCG hydrolysates might be a way to develop novel beverages with pleasant flavor profiles.

1. Introduction

Spent coffee grounds (SCG) is the solid residue from coffee brewing (with hot water and coffee grounds) in cafes or at home, and the production of instant soluble coffee powders in the industry (McNutt, 2019). Being the main residue of the coffee industry, it amounts to 6 million tons annually with rising trends (McNutt, 2019; Liu et al., 2021c). Despite being a waste, SCG still contains high amounts of carbohydrates, oils, and other high-value bioactive compounds (e.g. phenolic compounds, carotenoids, flavonoids), and is a potential source of energy (McNutt, 2019).

Recently, pre-treated SCG has been applied to develop alcoholic beverages with ethanol content ranging from 3 % to 10 % (v/v) fermented with monocultures of *Saccharomyces cerevisiae* and non-*Saccharomyces* yeasts (*Lachancea theromtolerans*, *Torulaspora delbrueckii* and *Pichia kluyveri*), and/or in combination with lactic acid bacteria (LAB, e.g. *Oenococcus oeni*) (Machado et al., 2018; Liu et al., 2021a, b, c). The fermentation process of alcoholic beverages includes alcoholic fermentation (AF) and sometimes, malolactic fermentation (MLF) (Matthews et al., 2004). Generally, AF positively enhances the production of aromatic profiles in the fermented SCG hydrolysates, with a significant volatile diversity from non-*Saccharomyces* yeasts such as *L. theromtolerans* (Liu et al., 2021a,b,c). MLF affects the flavor via changing the amount of lactic acid and some volatile constituents (e.g. diacetyl) (Balmaseda et al., 2018; Diez et al., 2010). Hence, yeasts, especially non-*Saccharomyces* yeasts, along with LAB play a vital role in the SCG hydrolysates fermentation.

The timing of inducing MLF is a key consideration in wine fermentation, co-inoculation (faster MLF) or sequential inoculation (slower MLF) of yeast and LAB. However, a number of drawbacks of co-inoculation was also reported such as off-flavor because of excess acetic acid formation and possible early death of yeast. In our previous study, we found early death of yeast in co-inoculation of *L. thermotolerans* Concerto and two *O. oeni* strains (Enoferm Beta and

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PN4) with a cell count inoculation ratio of around 1:100 (yeast : LAB) in SCG hydrolysates (Liu et al., 2021b). However, the co-inoculation of *L. thermotolerans* Concerto and *O. oeni* Lalvin 31 did not cause early death of yeast at a lower cell count inoculation ratio of around 1:5 (yeast : LAB).

Sequential inoculation of yeast and LAB may overcome the disadvantages of co-inoculation because LAB is introduced during or after the AF process. Numerus studies indicated that a later introduction of LAB into the fermented medium reduced the ethanol content, which meets the rising demand from the consumer's market (Vilela, 2020). The yeasts could release nutrients from autolysis to enhance LAB growth, boosting the malolactic activity and bacterial metabolism (Costello et al., 2008). Moreover, sequential inoculation of LAB after yeasts may exert positive effects on the aroma complexity, color preservation, and good flavour (e.g. fruity and lactic notes) (Balmaseda et al., 2018).

To ascertain whether *L. thermotolerans* Concerto and *O. oeni* Lalvin 31 are compatible at a high cell count inoculation ratio, this work evaluated and compared the growth and physicochemical changes of SCG hydrolysates during fermentation when *L. thermotolerans* Concerto and *O. oeni* Lalvin 31were co- and sequentially inoculated at the cell count inoculation ratio of around 1:100, extending our previous work (Liu et al., 2021b) mentioned above. The changes of non-volatile and volatile profiles of SCG hydrolysates with sequential inoculation were highlighted and compared with co-inoculation relative to respective mono-inoculations. This study may complement current efforts to develop novel SCG-derived alcoholic beverages.

2. Materials and methods

2.1. Production of SCG hydrolysates and precultures of microorganisms

The SCG hydrolysates were prepared by sequential acidic and enzymatic hydrolysis as reported previously (Liu et al., 2021c). Briefly, the collected SCG was first dried at 60 °C to less than 5 % (w/w). The dried SCG was then treated with hexane before being filtered, dried overnight and homogenized with deionized water (15 %, w/v). The aqueous mixture was further hydrolyzed under optimized conditions with citric acid (0.2 M, 121 °C, 1 h) and Viscozyme®L (6 %, w/v, 50 °C, 24 h) in succession.

The produced SCG hydrolysates were then cooled to ambient temperature before sucrose was added to adjust the °Brix reading to 15 (at pH 5.0) using a RX-5000a refractometer (ATAGO, Tokyo, Japan). Yeast extracts (0.25 g, Oxoid, Basingstoke, Hampshire, England) was used to supplement the SCG hydrolysates. The hydrolysate-sucrose mixture was then heated at 60 °C for 30 min. The pasteurization effectiveness was verified by enumeration using potato dextrose agar (PDA) (Oxoid, Basingstoke, UK), with added chloramphenicol (Danisco, Grindsted, Denmark) and modified MRS agar (Merck, Singapore) (Lu et al., 2018) containing Natamax (Danisco), for yeasts and bacteria respectively.

Precultures of yeast and LAB were prepared separately as described elsewhere (Liu et al., 2021b). Briefly, *L. thermotolerans* Concerto was cultured at 20 °C for 72 h in yeast nutrient broth (10 mL, pH 5.0) containing peptone, yeast extract, malt extract and glucose. Subsequently, 5 % (v/v) of activated yeast culture was propagated in the SCG hydrolysates incubated as above. Similarly, *O. oeni* Lalvin 31 was cultured in modified MRS broth (supplemented with 20 % apple juice, v/v, Marigold, Malaysia) for at 30 °C 4 days. Subsequently, 5 % (v/v) activated oenococcal-Lalvin culture was propagated in the SCG hydrolysates and incubated at 30 °C for 4 days.

2.2. Experimental design

Triplicate static fermentations (300 mL media in each 500 mL sterile flask) were conducted for each of the inoculation regimes: a) mono-inoculation of *L. thermotolerans* Concerto (yeast-Concerto, C) and *O. oeni* Lalvin 31 (oenococcal-Lalvin, L) with initial yeast of around 5 log CFU/mL and bacteria of around 7 Log CFU/mL, respectively; b) simultaneous inoculation of yeast-Concerto (~5 log CFU/mL) and oenococcal-Lalvin (~7 Log CFU/mL) defined as CL; c) sequential inoculation of yeast-Concerto (~5 log CFU/mL) on day 0 and oenococcal-Lalvin (~7 Log CFU/mL) on day 4 defined as SL. An uninoculated SCG hydrolysates blank was included and defined as C D0 at day 0 and CC D10 at day 10. All the flasks were incubated at 30 °C for 10 days. The physicochemical changes were studied through periodic sampling on days 0, 1, 2, 4, 7, and 10. Collected samples were kept at −20 °C before analysis.

2.3. Analysis of non-volatile profiles

Sugars, glycerol, alkaloids, organic and phenolic acids, were analyzed using HPLC (Shimazu, Kyoto, Japan) and amino acids were analyzed using an Amino Acid Analyzer (ARACUS, MembraPure, Berlin, Germany) with procedures detailed in Liu et al. (2021c). Ethanol was analyzed through an UFLC system (Agilent, Santa Clara, USA) connected to a RI detector, using 0.05 % (v/v) H_2SO_4 as the mobile phase at flow rate of 1.1 mL/min, going through a Supelcogel C-610 column (300 × 7.8 mm, Sigma-Aldrich, Barcelona, Spain) at 80 °C.

2.4. Analysis of volatile profiles

Volatiles were extracted using headspace-solid phase microextraction (HS-SPME) (Agilent, California, USA). The volatiles were analyzed through GC, with a flame ionization detector (FID) and mass spectrometer (MS), using helium as the carrier gas (Agilent, California, USA). Volatile compounds were identified through the NIST14 MS library and further confirmed through their linear retention indices (LRI). GC samples were adjusted to pH 2.5 with 1 M HCl before analysis. Butyl butyryl lactate (internal standard, 1 ppm in methanol) was added to 5 mL of the pH adjusted sample in a 20-mL GC vial to aid the identification and quantification of detected volatiles as presented in Liu et al. (2021c).

2.5. Statistical analysis

The data are expressed as the mean ± standard deviation. All data were subjected to Tukey's test and one-way analysis of variance (ANOVA) in SPSS 20.0® (SPSS Inc., Chicago, IL, USA), at a significance level of p value less than 0.05. Heatmaps were plotted using the R package Principal component analysis (PCA) was conducted for the volatiles using Matlab R2008a (MathWorks Inc., Natick, MA, USA).

3. Results and discussion

3.1. Cell population kinetics

The yeast and LAB respectively displayed a similar growth trend in different fermentation regimes from day 0 to day 10 except for simultaneous inoculation (CL samples) (Fig. 1a, b). From day 0 to day 1, the yeast cell population increased from 5.09 Log CFU/mL to around 6.55–6.84 Log CFU/mL for all treatments involving yeast-Concerto (SL, C, and CL samples). Subsequently, the yeast cell population gradually decreased to 5.14–5.28 Log CFU/mL in SL and C samples at day 10. Interestingly, the cell population kinetics of yeast-Concerto in SL samples followed a similar trend to that of mono-inoculation (C), but was distinctly different from that of co-inoculated CL samples, in which the yeast was undetectable by day 7 (Fig. 1a).

The cell population of oenococcal-Lalvin in SL samples increased from 7.31 Log CFU/mL to around 7.76 Log CFU/mL, which was lower than that in both mono-inoculation (L, 8.65 Log CFU/mL) and simultaneous inoculation (CL, 8.60 Log CFU/mL) (Fig. 1b). The lower growth of oenococcal-Lalvin in SL samples might be due to the insufficient nutrients in the medium and the increasing content of ethanol (Vilela, 2020).

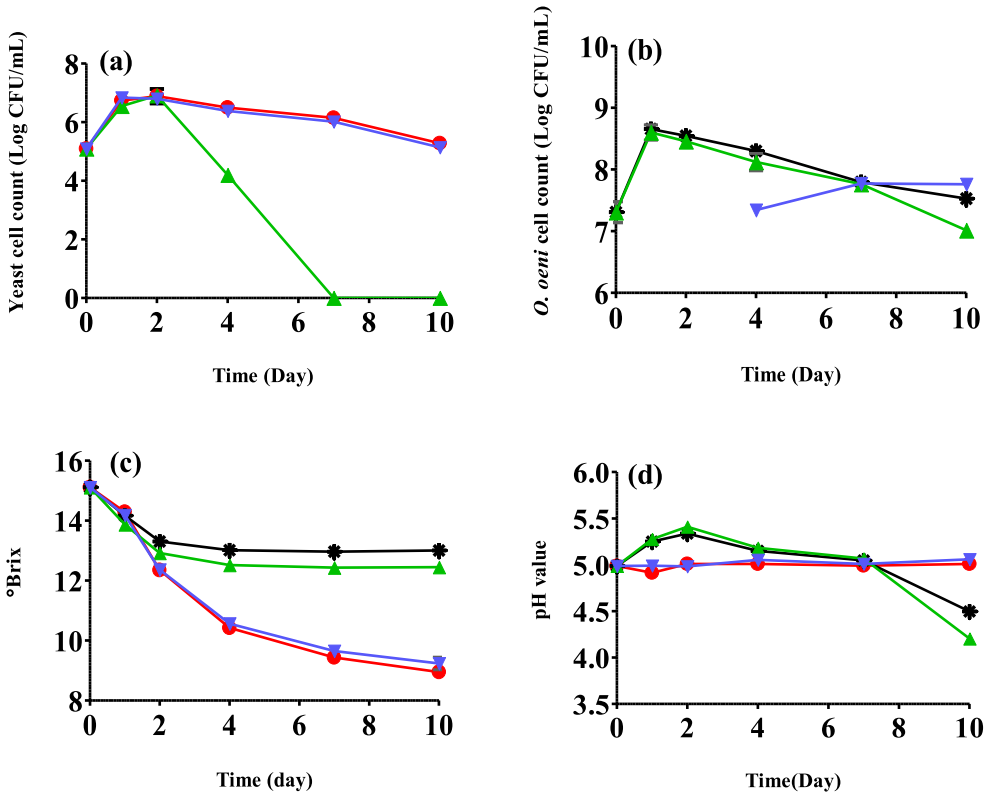

Fig. 1. Population kinetics of *L. thermotolerans* Concerto (a) and *O. oeni* Lalvin 31 (b), changes of °Brix (c) and pH (d) during SCG hydrolysates fermentation by yeast-Concerto and/or oenococcal-Lalvin. (●): Yeast-Concerto mono-inoculation; (※): Oenococcal-Lalvin mono-inoculation; (▲): Simultaneous inoculation of yeast-Concerto and oenococcal-Lalvin; (▼): Sequential inoculation of yeast-Concerto and oenococcal-Lalvin.

The early cell death of yeast-Concerto and lower final oenococcal-Lalvin cell population in CL samples could be ascribed to the inhibitory interactions between yeast and bacteria. For example, oenococcal-Lalvin may produce some yeast incompatible metabolites such as acetic acid (Kantachote et al., 2010; Zhao and Fleet, 2015), which was the highest concentration in CL samples (3.70 g/L), relative to the other treatments (Table 1). This observation did not align with Liu et al. (2021b), where it was found that oenococcal-Lalvin was compatible with yeast-Concerto during SCG hydrolysates fermentation, where yeast-Concerto maintained a relatively stable, high cell population of approximately 6 Log CFU/mL at the end of fermentation. The reason could be due to the different initial inoculation amount of *O. oeni* in different studies. In the present study, a relatively higher initial cell population of *O. oeni* (7.31 Log CFU/mL), relative to yeast-Concerto (5.09 log CFU/mL) used, as compared to Liu et al. (2021b), where a relatively lower initial population ratio of oenococcal-Lalvin (6.5 Log CFU/mL) and yeast-Concerto (5.5 Log CFU/mL) was used. The higher inoculation of oenococcal-Lalvin could have resulted in a greater competition with the yeast for nutrients and also produced more inhibitory metabolites to yeast. Interestingly, yeast and LAB co-grew well in the SCG hydrolysates when oenococcal-Lalvin was inoculated on day 4 (Fig. 1a, b). Therefore, sequential inoculation of LAB might be an effective way to avoid the early death of yeast-Concerto in SCG hydrolysates fermentation, although oenococci had limited growth.

3.2. Changes in total soluble solids, sugars and ethanol

The changes in total soluble solids, sugars and ethanol are shown in Figs. 1c, Fig. 2a–g, and Table 1 respectively. As displayed in Fig. 1c, the °Brix value gradually decreased in SL and C samples during fermentation with a significant decline from day 0 to day 4, while the °Brix of mono- and co-inoculation samples (L and CL) remained relatively stable since day 4.

The overall total sugar contents showed a significant decreasing trend (Fig. 2g and Table 1), with the highest total sugar utilization in SL samples (△ = 110.30 g/L), followed by C (△ = 108.22 g/L), CL (△ = 69.01 g/L) and L (△ = 48.01 g/L) samples, corresponding to the changes in °Brix (Fig. 1c). Additionally, all sugars, with the exception of arabinose (Fig. 2f), were significantly utilized by yeast-Concerto and/or oenococcal-Lalvin during fermentation (Table 1). In general, the catabolism levels of fructose (Fig. 2a), glucose (Fig. 2b), sucrose (Fig. 2c), galactose (Fig. 2d) and mannose (Fig. 2e) in SL samples were largely similar to C samples, but higher than those in CL and L samples (Table 1). The lower sugar metabolism in CL samples could be ascribed to the inhibitory effect from LAB, causing the early yeast cell death (Zhao and Fleet, 2015).

This variation in total soluble solids and sugar contents partially translated to a corresponding variation in the production of ethanol (Fig. 2h) and glycerol (Table 1). The ethanol content in SL samples was 4.91 % (v/v), which was a bit lower than that in C samples (5.56 % v/v) but significantly higher than that of CL (1.77 % v/v) and L (undetectable) samples, emphasizing that sequential inoculation of oenococcal-Lalvin facilitated ethanol generation by the yeast (Englezos et al., 2019). The higher ethanol production in C and SL samples could be due to a similar extent of glucose metabolism from 71.89 g/L to 4.13 g/L (C samples) and 4.06 g/L (SL samples) respectively and thus a similar translation to ethanol as compared to the lower ethanol content in CL samples, corresponding to a higher final glucose content (29.38 g/L). The low sugar utilization rate in CL samples could be due to the inhibitory effect of oenococci, given the early yeast death. In addition, the relatively higher glycerol content in SL samples possibly provided for a balanced fermentative mixture with desirable mouthfeel and flavor intensity.

Table 1
Physicochemical properties of unfermented and fermented SCG hydrolysates.

	Unfermented SCG hydrolysates		Fermented SCG hydrolysates at day 10			
	C D0	CC D10	C	L	CL	SL
pH	4.99 ± 0.01c	4.97 ± 0.03c	5.01 ± 0.00cd	4.50 ± 0.03b	4.20 ± 0.01a	5.06 ± 0.02d
°Brix (%)	15.11 ± 0.01e	15.47 ± 0.00f	8.95 ± 0.04a	13.01 ± 0.02d	12.45 ± 0.04c	9.23 ± 0.21b
Ethanol (%, v/v)	0.00 ± 0.00a	0.00 ± 0.00a	5.56 ± 0.08d	0.00 ± 0.00a	1.77 ± 0.02b	4.91 ± 0.13c
Glycerol (g/L)	0.00 ± 0.00a	0.00 ± 0.00a	0.30 ± 0.01c	0.00 ± 0.00a	0.16 ± 0.00b	0.48 ± 0.01d
Sugars (g/L)						
Fructose	18.53 ± 0.20c	19.51 ± 0.93c	4.74 ± 0.24a	6.91 ± 0.07b	5.43 ± 0.22a	6.80 ± 0.12b
Glucose	71.89 ± 1.64d	72.19 ± 1.33d	4.13 ± 0.23a	42.21 ± 1.05c	29.38 ± 0.40b	4.06 ± 0.33a
Sucrose	8.32 ± 0.14e	7.91 ± 0.61e	2.52 ± 0.09b	5.77 ± 0.10d	5.08 ± 0.27c	2.28 ± 0.01a
Mannose	23.92 ± 0.30c	23.45 ± 0.95c	12.31 ± 0.15a	22.45 ± 0.09c	16.02 ± 0.84b	11.68 ± 0.36a
Galactose	23.21 ± 0.37d	23.31 ± 0.67d	13.01 ± 0.77b	19.63 ± 0.38c	20.07 ± 0.66c	9.92 ± 0.73a
Arabinose	2.54 ± 0.05a	2.57 ± 0.04a	2.47 ± 0.05a	2.43 ± 0.14a	2.41 ± 0.31a	2.37 ± 0.28a
Total	147.40 ± 0.62d	146.91 ± 2.94d	39.18 ± 1.11a	99.39 ± 1.50c	78.39 ± 1.05b	37.10 ± 1.07a
Organic acids (g/L)						
Citric acid	38.40 ± 3.25c	38.33 ± 0.89c	38.34 ± 2.40c	0.30 ± 0.00a	0.30 ± 0.00a	34.34 ± 0.43b
α-Ketoglutaric acid (mg/L)	50.21 ± 0.25a	56.90 ± 3.38b	50.12 ± 0.46a	52.74 ± 0.94ab	50.90 ± 0.77a	50.15 ± 1.22a
L-Malic acid	0.38 ± 0.02c	0.39 ± 0.01c	0.15 ± 0.08b	0.24 ± 0.01b	0.23 ± 0.01b	0.15 ± 0.00a
Pyruvic acid	0.20 ± 0.01c	0.18 ± 0.00bc	0.38 ± 0.02d	0.16 ± 0.00b	0.13 ± 0.08a	0.35 ± 0.01d
Succinic acid	2.90 ± 0.18a	2.89 ± 0.17a	3.16 ± 0.10a	22.92 ± 0.18c	23.58 ± 0.54c	9.02 ± 0.22b
Lactic acid	0.32 ± 0.00a	0.43 ± 0.01a	1.08 ± 0.03b	26.13 ± 0.28e	24.97 ± 0.58d	4.30 ± 0.42c
Acetic acid	0.29 ± 0.01a	0.30 ± 0.01a	0.39 ± 0.01b	2.49 ± 0.03c	3.70 ± 0.12c	2.48 ± 0.09c

Note: Different letters of a, b, c, d, e, f stands for the significant differences between samples ($p < 5\%$). (C D0): unfermented SCG at day 0; (CC D10): unfermented SCG at day 10; (C): Yeast-Concerto mono-inoculation; (L): Oenococcal-Lalvin mono-inoculation; (CL): Simultaneous inoculation of yeast-Concerto and oenococcal-Lalvin; (SL): Sequential inoculation of yeast-Concerto and oenococcal-Lalvin.

3.3. Changes in pH and organic acids components

The changes in the overall trend of pH for SL samples remained relatively constant (pH 4.99–5.06) during fermentation, following a similar trend to C samples (Fig. 1d, Table 1). However, significant decreases in pH were detected in L and CL samples, where pH decreased to 4.50 and 4.20 respectively at the end of fermentation. It could be ascribed to the higher production of lactic, succinic and acetic acids during fermentation (Fig. 3). Our results agreed with the changes of organic acids along with pH values observed in mixed pear fruit wine (Tsegay, 2020).

During fermentation, the changes of organic acids displayed two types of trends except for malic acid with similar declining trends in all treatments (Fig. 3). Some organic acids (e.g. citric acid, malic acid, pyruvic acid) in SL samples presented a similar trend to that of C samples while other organic acids (e.g. lactic acid, succinic acid and acetic acid) presented obviously different trends. The high initial level of citric acid was because of the use of this acid in the preparation of SCG hydrolysates. Citric acid significantly decreased in SCG hydrolysates with inoculation of oenococcal-Lalvin, with especially a sharp decrease from 38.40 g/L to 0.30 g/L in L and CL samples (Fig. 3a, Table 1). This could be ascribed to the high activity of citric acid catabolism by oenococci (Swiegers et al., 2005). In contrast, a significantly higher level of citric acid remained in SL samples although oenococcal-Lalvin was inoculated at day 4, which could be due to a shorter period for metabolism of citric acid, as well as the lower cell counts of oenococcal-Lalvin (Fig. 1b).

Interestingly, an increase in pyruvic acid was observed in SL and C samples, while a slight decrease was observed in L and CL samples (Fig. 3c, Table 1). The increase of pyruvic acid in SL and C samples could be accounted for by endogenous production by yeast-Concerto during AF while the decrease in L and CL samples could be explained by its reduction to lactic acid by oenococcal-Lalvin (Vilela, 2019). Pyruvic acid could help *O. oeni* increase its MLF performance by acting as an external electron acceptor, allowing NAD^+ to be regenerated rapidly (Maicas et al., 2002). Besides, it could also promote the production of diacetyl (Balmaseda et al., 2018).

Although significant increases in lactic, acetic and succinic acids were observed in L, CL, SL samples during fermentation (Fig. 3d, e, 3f), the amount of lactic, acetic and succinic acids in SL samples was significantly lower than those in L and CL samples. The higher production of acetic and lactic acids than those reported in Liu et al. (2021b) could be ascribed to the higher oenococcal-Lalvin inoculation ratio, and therefore oenococci metabolized more citric acid and sugars to acetic acid, lactic acid and CO_2, as well as minor levels of diacetyl and acetoin (Mendes Ferreira et al., 2020). The early introduction of oenococcal-Lalvin in CL samples contributed to the higher levels of acetic and lactic acids, which could be attributed to the heterofermentative nature of oenococci and breakdown of citric acid (Liu et al., 2021b; Tristezza et al., 2016). In turn, the later introduction of oenococcal-Lalvin in SL samples could efficiently limit the production of lactic and acetic acids (Fig. 3d, e).

A moderate increase of succinic acid (9.02 g/L) was found in SL samples as compared to a sharp increase in L and CL samples (22.92 g/L, 23.58 g/L) (Fig. 3e, Table 1). Succinic acid could be synthesized by both yeasts (through the tricarboxylic acid (TCA) cycle, glyoxylate cycle and amino acid catabolism) and LAB (through the reductive pathway of TCA cycle from citric acid metabolism), with a higher contribution by the later (Mendes Ferreira et al., 2020). The moderate level of succinic acid in SL samples has potential to improve the sensory/mouthfeel character of SCG hydrolysates beverages due to its sour, salty, and bitter characteristics (Lu et al., 2017). This acid could also confer possible health benefits like improvements in metabolic performance (Giorgi-Coll et al., 2017). Excessive quantities in L and CL samples, however, may negatively affect the taste of the final product (Vilela, 2019).

3.4. Changes in phenolic acids, alkaloids and antioxidant capacity

The changes in alkaloids and phenolic acids are shown in Fig. 4a and Fig. 4b respectively. In general, some alkaloids (e.g. theobromine and theophylline) decreased significantly while some phenolic acids (e.g. ferulic acid and *p*-coumaric acid) increased significantly after fermentation. It is interesting to note that a significant decrease of caffeine was only observed in SL samples (Fig. 4a). A previous study showed that both

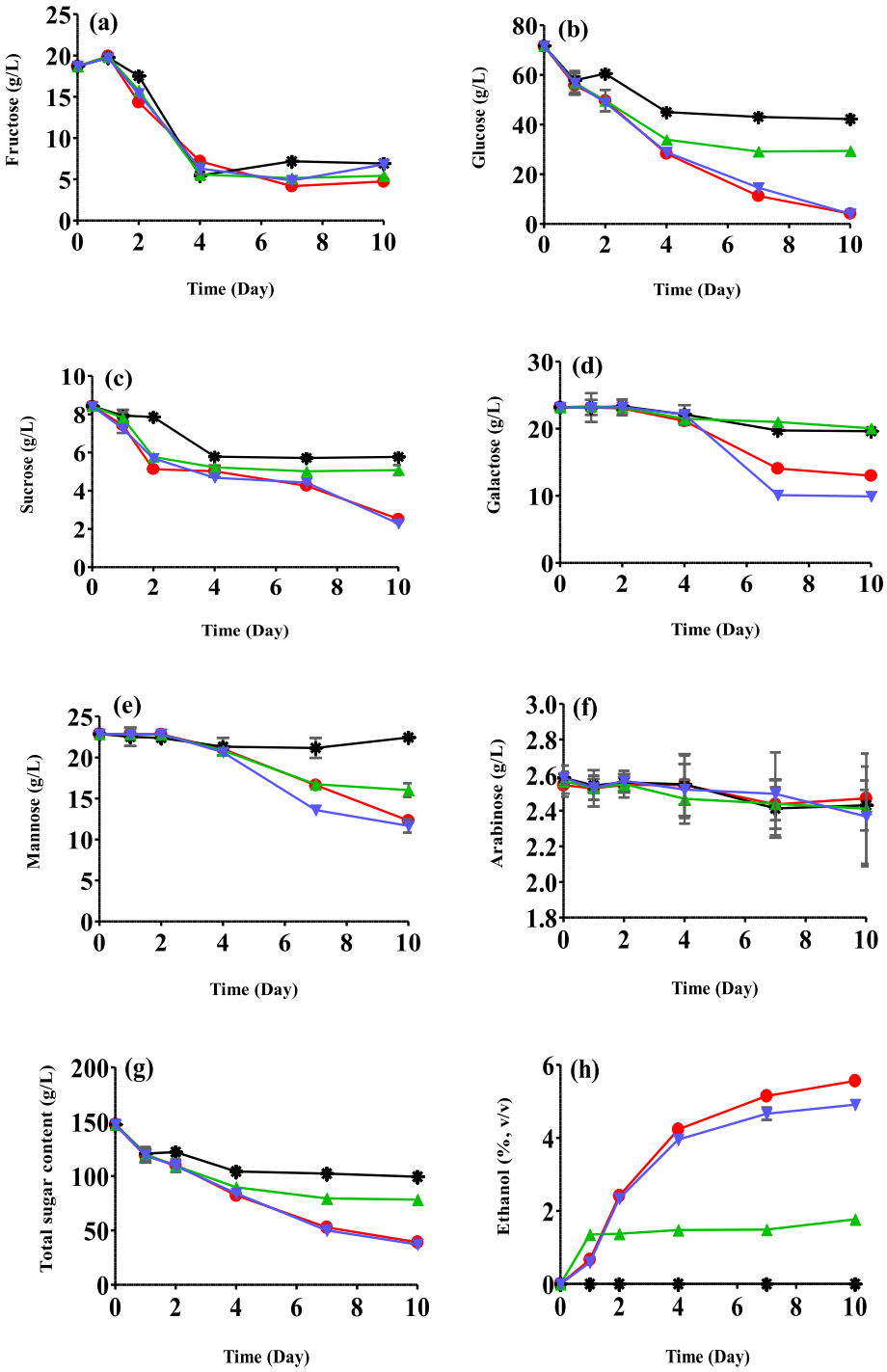

Fig. 2. Changes of sugars (a–g) and production of ethanol (h) during SCG hydrolysates fermentation by yeast-Concerto and/or oenococcal-Lalvin. (●): Yeast-Concerto mono-inoculation; (※): Oenococcal-Lalvin mono-inoculation; (▲): Simultaneous inoculation of yeast-Concerto and oenococcal-Lalvin; (▼): Sequential inoculation of yeast-Concerto and oenococcal-Lalvin.

yeasts and LAB were able to decrease caffeine content by absorbing or degrading caffeine due to its structural similarity to the purine molecule (Purwoko et al., 2022). In addition, caffeine in SL samples could have also been degraded to theobromine and theophylline by yeast through *N*-demethylation (Hakil et al., 1998). The insignificant changes of caffeine in C, L and CL samples could be due to a balance between its degradation and generation.

Theoretically, the contents of theobromine and theophylline should increase especially in SL samples due to a net degradation of caffeine. However, their significant decreases might be because they were degraded by yeasts, leading to the formation of compounds like 3-methylxanthine (through 7- and 1-demethylation, respectively) (Hakil et al., 1998).

In addition, chlorogenic acids in SL samples displayed a significant decrease while *p*-coumaric acid showed a significant increase, which followed a similar trend to those in control C, and CL samples. The increase in *p*-coumaric acid could be due to the hydrolysis of chlorogenic acids into quinic acid and other phenolic acids like caffeic, *p*-coumaric,

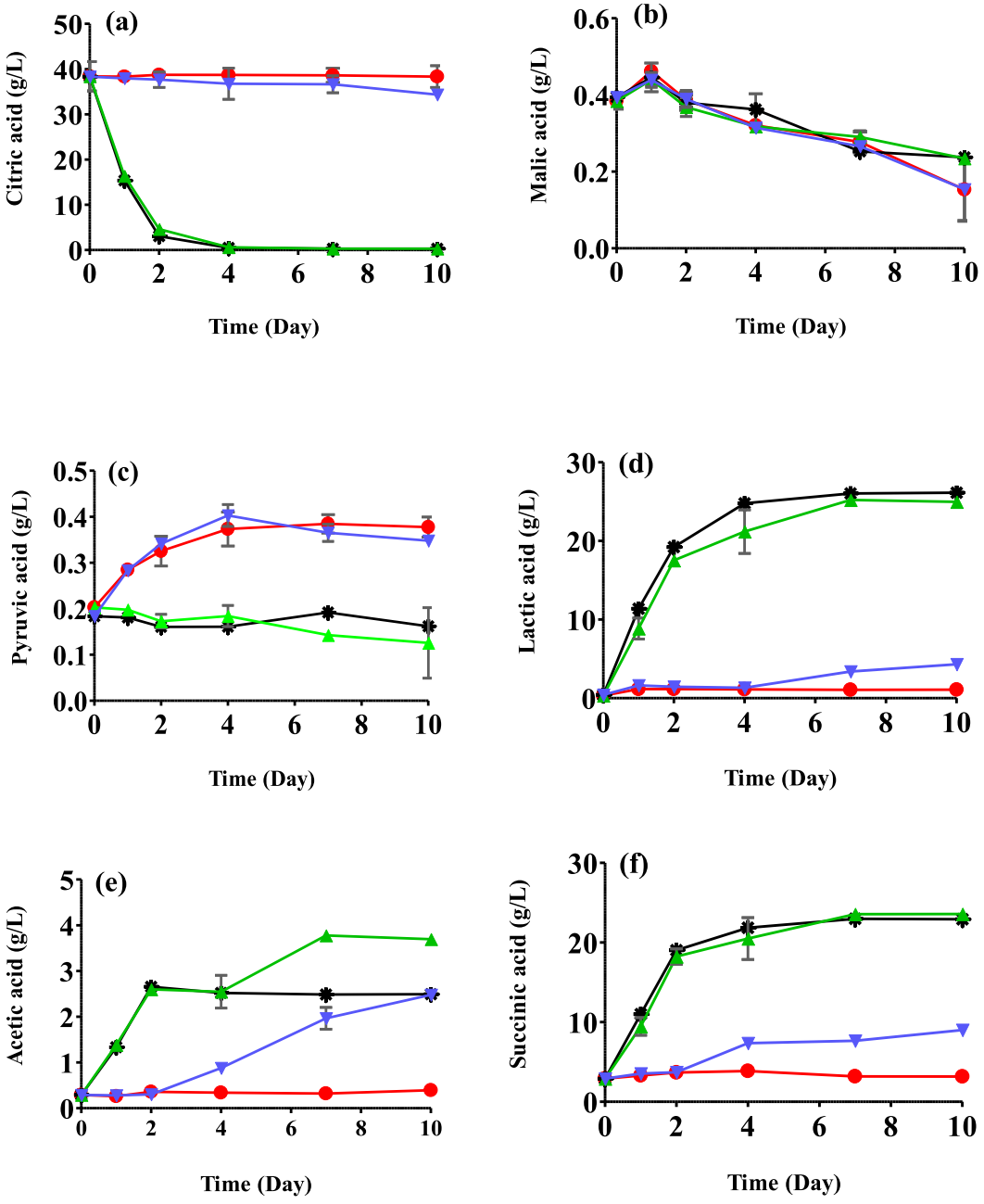

Fig. 3. Changes of organic acids (a–f) during SCG hydrolysates fermentation by yeast Concerto and/or oenococcal-Lalvin. (●): Yeast-Concerto mono-inoculation; (※): Oenococcal-Lalvin mono-inoculation; (▲): Simultaneous inoculation of yeast-Concerto and oenococcal-Lalvin; (▼): Sequential inoculation of yeast-Concerto and oenococcal-Lalvin.

and ferulic acids by microbial esterases (e.g. cinnamoyl esterases) produced by *O. oeni* (Collombel et al., 2019) or by chlorogenic acid esterases (e.g. dimeric cinnamate esterase), which had been reported in other species of fungi (e.g. *Aspergillus* spp.) (Asther et al., 2005). Ferulic acid also significantly increased in all treatments at the end of fermentation (Fig. 4b). However, different from other phenolic acids, caffeic acid showed a significant decrease in L, CL, and SL samples, while no significant change was observed in C samples, suggesting a possible degradation by oenococcal-Lalvin. Several previous studies reported LAB might be able to metabolize caffeic acid to dihydrocaffeic acid by phenolic acid reductase (Wu et al., 2020), or convert it to volatile phenols such as 4-vinylcatechol (Bel-Rhlid et al., 2013).

The changes in antioxidant capacity are shown in Fig. 4c (ORAC), Fig. 4d (DPPH) and Fig. 4e (TPC), respectively. Interestingly, no significant differences were detected for ORAC, DPPH and TPC values before and after fermentation among all samples, suggesting the fermented samples might have similar antioxidant capacities to unfermented samples. One possible reason was the establishment of a balance, in terms of antioxidant potential, between the significant increase in ferulic and *p*-coumaric acids and decrease in alkaloids and other phenolic acids (e.g. chlorogenic acids and caffeic acid). Another reason might be that the changes in alkaloid and phenolic acid contents were insufficient to bring about significant changes in measured antioxidant capacities.

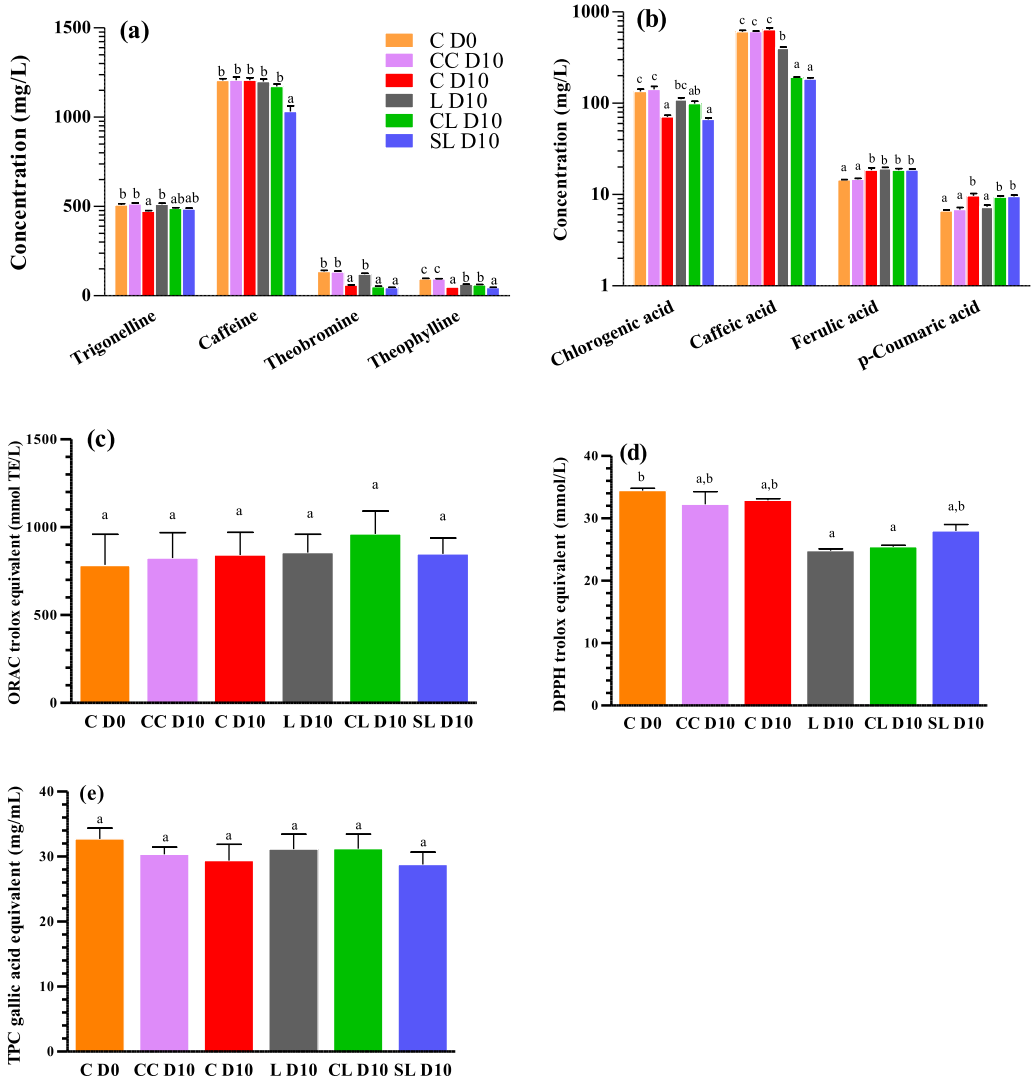

Fig. 4. Changes of alkaloids (a), phenolic acids (b), and antioxidant capacity of SCG hydrolysates before and after fermentation by yeast-Concerto and/or oenococcal-Lalvin, ORAC (c), DPPH (d) and TPC (e). CD0: unfermented SCG hydrolysates at day 0; CCD10: unfermented SCG hydrolysates at day 10; CD10: Yeast-Concerto mono-inoculation at day 10; LD10: Oenococcal-Lalvin mono-inoculation at day 10; CLD10: simultaneous inoculation of yeast-Concerto and oenococcal-Lalvin at day 10; SLD10: sequential inoculation of yeast-concerto and oenococcal-Lalvin at day 10.

3.5. Changes in amino acid profiles

With yeast extract supplementation, YAN in unfermented SCG hydrolysates reached up to159.33 mg N/L (Table S3), which is higher than that requested in grape wine (≥140 mg N/L) fermentation (Gobert et al., 2019), indicating adequate YAN for fermentation in SCG hydrolysates. The initial YAN (159.33 mg N/L) decreased to 72.17 mg N/L (C), 95.99 mg N/L (L), 92.29 mg N/L (SL) and 115.24 mg N/L (CL) after fermentation, indicating YAN was consumed in all fermentation regimes. The relatively higher utilization of YAN in SL and C samples could be due to the postponed or lack of inhibitory actions from oenococci. The higher utilization of YAN in SL samples than that of CL samples could be accounted for by the co-growth of yeast and bacteria in the former as compared to the early yeast cell death in CL samples.

The changes of free amino acid profiles across all different fermentation regimes are presented in Fig. 5 and Table S3. After fermentation, a significant decrease in most amino acids was observed except for serine (a significant decrease for all treatments except for L samples), glycine (no significant decrease for all treatments), alanine (only a significant decrease for CL samples) and valine (only a significant decrease for C and L samples). Compared to CL samples, SL samples displayed significantly lower residual concentrations of glutamic acid, aspartic acid, alanine, cysteine, and ammonium, indicating a higher utilization rate. These amino acids could be used for the cell growth and increase in biomass, as well as the generation of volatile flavor compounds such as carbonyls, (higher) alcohols (from amino acids, leucine, valine, isoleucine, phenylalanine), esters and sulfur compounds (from amino acids, methionine, cysteine) by both yeast and LAB (Fairbairn et al., 2017; Swiegers et al., 2005).

3.6. Changes in volatile profiles and PCA plot

Volatile compounds detected in SCG hydrolysates prior to and after fermentation are shown in Fig. 6 and Table S4. In general, 69 compounds were identified, including 11 acids, 5 alcohols, 6 aldehydes, 16 esters, 5 furans, 7 ketones, 4 pyrazines, 4 pyrroles, 3 terpenoids, and 8 volatile phenols. Besides the existing aroma compounds in unfermented SCG hydrolysates, some newly produced volatiles (e.g. esters, aldehydes and furans) were also detected.

Decreases in concentrations of most volatile acids (e.g. short and

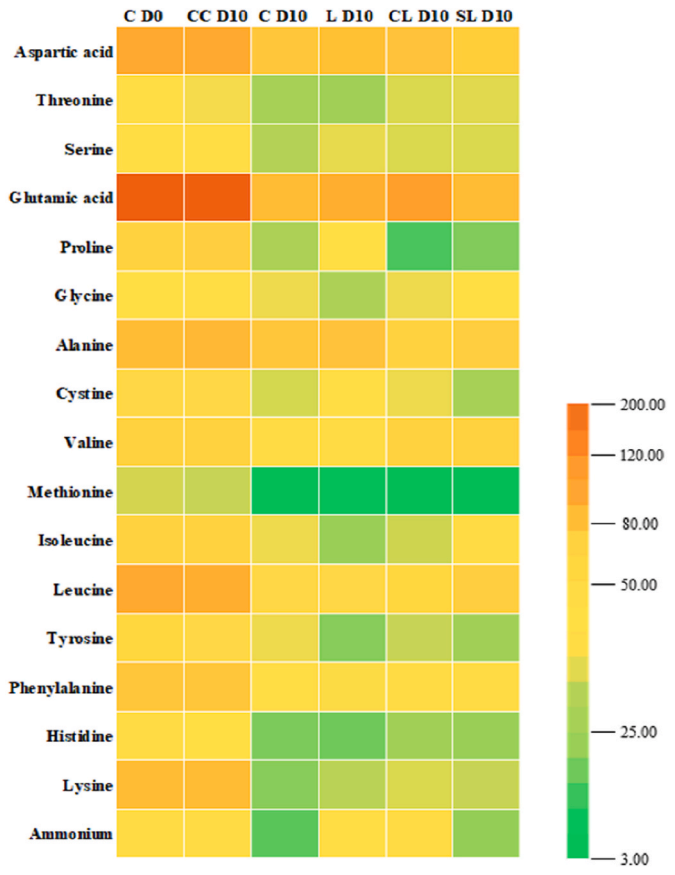

Fig. 5. Heatmap of changes of amino acids and ammonia in SCG hydrolysates before and after fermentation by yeast-Concerto and/or oenococcal-Lalvin. (C D0): unfermented SCG hydrolysates at day 0; (CC D10): unfermented SCG hydrolysates at day 10; (C): Yeast-Concerto mono-inoculation; (L): Oenococcal-Lalvin mono-inoculation; (CL): Simultaneous inoculation of yeast-Concerto and oenococcal-Lalvin; (SL): Sequential inoculation of yeast-Concerto and oenococcal-Lalvin. Unit of scale bar is mg/L.

medium chain fatty acids) were observed, while the content of 4-hexenoic acid, octanoic acid, decanoic acid, and benzoic acid significantly increased (Fig. 6; Table S4). In general, the decrease of volatile acids could be because they were used as precursors of esterification and/or alcoholysis especially in SL and CL samples, in which the lower levels of hexanoic acid corresponded to the higher levels of ethyl hexanoate (Fig. 6; Table S4). The increases in 4-hexenoic acid, octanoic acid, decanoic acid, and benzoic acid would be a balance of the production and utilization of volatile acids (Mendes Ferreira et al., 2020).

Among all aldehydes detected, furfural and 5-methylfurfural significantly increased in SL samples (Fig. 6; Table S4), which might be released from its bound form in SCG hydrolysates during fermentation, giving a sweet, caramel-like aroma (Liu et al., 2021b). However, the concentrations of most other aldehydes remarkably decreased (Fig. 6; Table S4). For example, compounds contributing to the characteristic coffee aroma such as 5-methyl-2-furancarboxaldehyde and benzaldehyde that were found in high concentrations in the unfermented SCG hydrolysates were partially metabolized, possibly being reduced to their respective (higher) alcohols such as benzyl alcohol or oxidized to their respective acids such as benzoic acid (Liu et al., 2021b; Zapata et al., 2018). In addition, SL samples contained higher levels of 5-methylfurfural than that of CL samples, while other aldehydes were in similar amounts in SL and CL samples.

Alcohols could be generated from the reduction of aldehydes, metabolism of amino acids and other substrates, besides sugars. The concentration of alcohols increased with the exception of 2-heptanol (Fig. 6; Table S4). Specifically, 5-methylfurfuryl alcohol, isoamyl alcohol and 2-phenylethyl alcohol were produced in SL samples with a lower amount as compared to C and CL samples. 5-Methylfurfuryl alcohol might be formed from reduction of its aldehyde or released from SCG hydrolysates since this compound originated from the Maillard reaction between hexose/pentose and theanine (Baert et al., 2012). The varied amount of isoamyl alcohol and 2-phenylethyl alcohol in SL, C, and CL samples was likely produced from leucine and phenylalanine respectively via the Ehrlich pathway by yeasts (Lu et al., 2016). The less production of isoamyl alcohol and 2-phenylethyl alcohol in SL samples, as compared to C samples, could be due to the early death or less growth of yeast-Concerto. Additionally, the highest amount of benzyl alcohol was found in SL samples, and it could be formed from the reduction of benzaldehyde. It has been reported that benzyl alcohol may impart a rose-like aroma and 5-methylfurfuryl alcohol could contribute a sweet, caramel-like aroma (Lu et al., 2016, 2018; Liu et al., 2021b; Kim et al., 2019).

Esters are significant aroma-impact odorants. In general, the 16 esters were mainly generated from either the metabolism of yeast-Concerto or the metabolism of oenococci (Fig. 6; Table S4), with most types of esters in SL samples. Ethyl esters such as ethyl (*Z*)-4-hexenoate, (*E,E*)-ethyl 2,4-hexadienoate, ethyl octanoate, ethyl (2)-octanoate, and ethyl nonanoate were all formed from the esterification of ethanol and the respective volatile fatty acids, whereas isoamyl acetate was formed mostly by the alcoholysis of isoamyl alcohol under the action of acetyl-CoA. The synthesis of acetyl esters using alcohols and acetyl-CoA was catalyzed by alcohol acetyltransferases (Swiegers et al., 2005). Although the number of esters in CL and SL samples was the same, a higher concentration of most esters was found in SL samples as compared to that in CL samples, ascribing to the bacterial inhibitory effects on the yeast-Concerto in CL samples (Fig. 1). Besides esters from yeast-Concerto, esters like methyl acetate and methyl lactate were detected in samples involving oenococcal-Lalvin with similar amounts in SL and CL samples.

Overall, the full range of esters was only detected in SL and CL samples, and a significantly higher concentration of most esters (e.g. ethyl acetate, ethyl hexanoate and ethyl 2-octenoate) was detected in SL samples. This highlighted the advantage of adopting a sequential inoculation method to generate and retain a wide range of esters, at enhanced concentrations.

Variations in furans presented two trends (Fig. 6; Table S4). 3-Acetyl-2,5-dimethylfuran (SL > CL), 2-methoxyfuran (SL > CL) and 2-furfuryl-5-methylfuran (SL < CL) increased, while 2-pentylfuran and 2-acetyl-5-methylfuran decreased after fermentation. Furans are mainly formed from the oxidative degradation of polyunsaturated fatty acids, where an increase in concentration of 3-acetyl-2,5-dimethylfuran (in SL, CL and L samples), and 2-furfuryl-5-methylfuran (in all treatments) was observed.

Pyrazines and pyrroles impart the coffee, nutty and roasted flavors in coffee and SCG (Liu et al., 2021b; Mortzfeld et al., 2020). The concentration of most pyrazines and pyrroles decreased across the fermentation where the extent of decrease varied among different fermentation regimes (Fig. 6; Table S4). After fermentation, the retention of 2,5-dimethylpyrazine and ethenylpyrazine in SL samples was higher than those in CL samples.

The concentration of most terpenoids and volatile phenols decreased across the fermentations with the exception of 4-ethylguaiacol (Fig. 6; Table S4). 4-Ethylguaicol could be produced from hydroxycinnamic and vinylphenol precursors including *p*-coumaric and ferulic acids through yeast and LAB metabolism, and the changes in concentration of volatile phenols depended on both the strains of yeast used and the coumaric acid decarboxylase, vinylphenol reductase and cinnamoyl esterase activities of the LAB utilized (Swiegers et al., 2005; Virdis et al., 2021).

Overall, across the different fermentation regimes, SL samples retained and/or generated the widest variety of volatile compounds (especially desirable esters and ketones), making it favorable for the development of more complex and aromatic fermented SCG beverages.

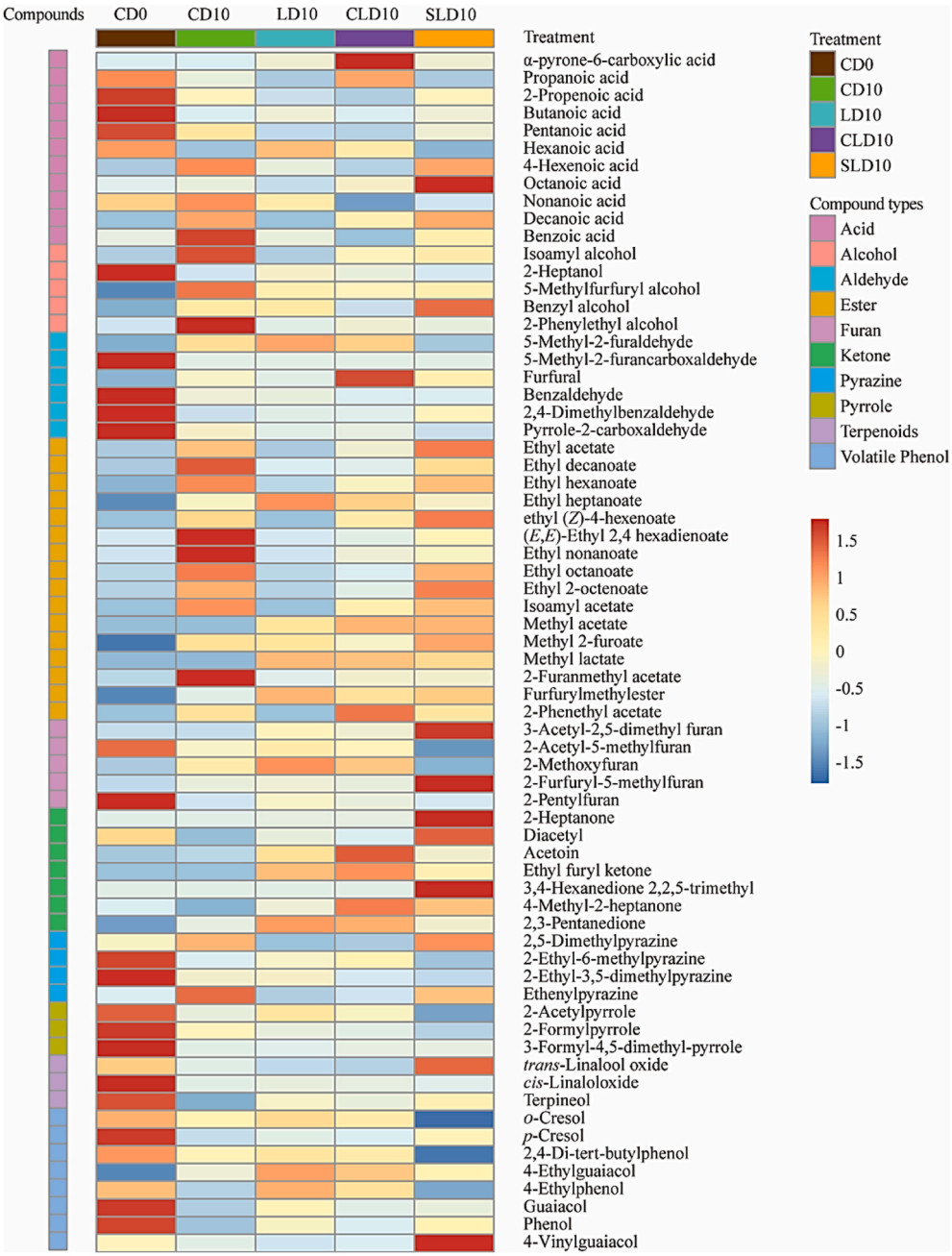

Fig. 6. Heatmap of volatile profiles of SCG hydrolysates before and after fermentation by yeast-Concerto o and/or oenococcal-Lalvin. (C): Yeast-Concerto mono-inoculation; (L): Oenococcal-Lalvin mono-inoculation; (CL): Simultaneous inoculation of yeast-Concerto and oenococcal-Lalvin; (SL): Sequential inoculation of yeast-Concerto and oenococcal-Lalvin; (D0): day 0; (D10): day10.

PCA was performed using key volatile components from Table S4, as well as acetic acid and ethanol from Table 1, to show the effects of different fermentation regimes on chemical composition of SCG hydrolysates (Fig. 7). The first two principal components (PCs) comprised 88.44 % of the total variance (Fig. 7). PC1 accounted for 56.17 % of the total variance and separated SL and C samples from L and CL samples. This could be because the former two treatments contained more ethyl 2-octenoate, ethyl (*Z*)-4-hexenoate, ethyl acetate, methyl 2-furoate, 2-furfuryl-5-methylfuran, 4-vinylguaiacol, 2-heptanone, octanoic acid, diacetyl, 3-acetyl-2,5-dimethyl furan, ethanol than that in CL and L samples. PC2 accounted for 32.27 % of the total variance and distinguished SL and L samples from CL and C samples, which contained higher contents of 2-phenylethyl alcohol, benzaldehyde, 2-furanmethyl acetate, ethyl nonanoate, (*E,E*)-ethyl 2,4-hexadienoate, ethyl decanoate, isoamyl alcohol, ethyl hexanoate, ethenylpyrazine, and ethyl octanoate.

4. Conclusion

Different inoculation regimes (mono-, co- and sequential inoculations) of *L. thermotolerans* Concerto and *O. oeni* Lalvin 31 were evaluated in this study. Sequential inoculation showed a stable growth of oenococcal-Lalvin and prevented the early yeast death as compared to co-inoculation. Adequate ethanol production and desirable levels of glycerol were observed in sequential inoculation. Relatively lower levels of undesirable acetic, lactic, and succinic acids were detected in sequential samples than that in co-inoculated samples as well as both monocultured samples, which may reduce the apparent sour, salty, and bitter taste of the fermented mixtures. Moreover, sequential samples

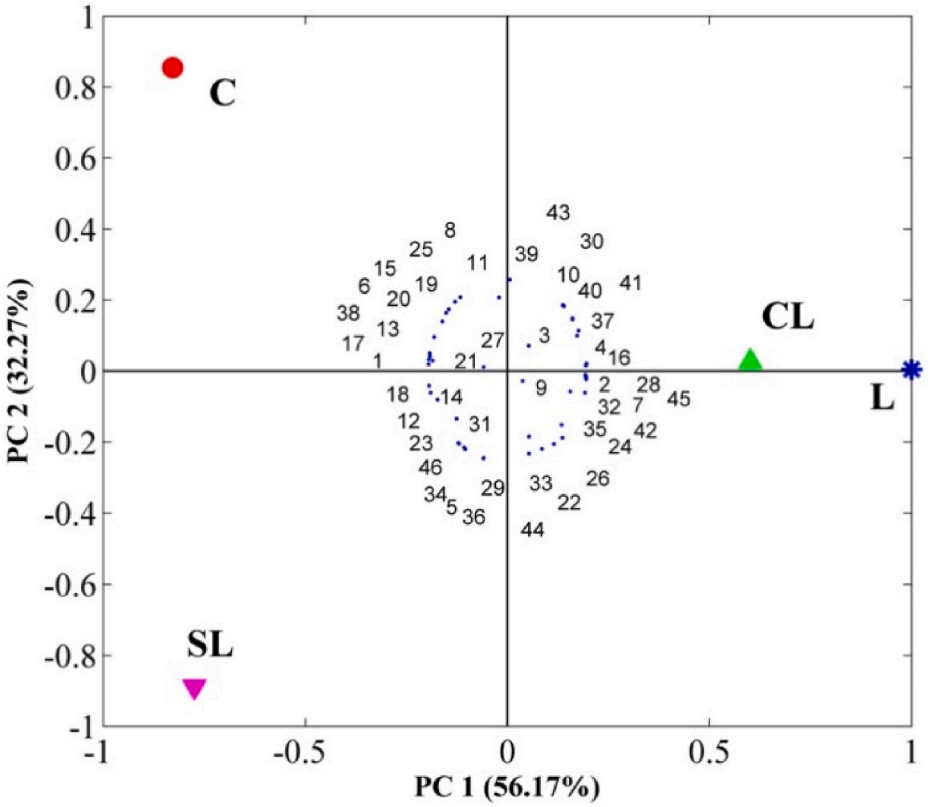

Fig. 7. Bi-plot of PCA of 46 volatile compounds (Table S5) in fermented SCG hydrolysates. (C): Yeast-Concerto mono-inoculation; (L): Oenococcal-Lalvin mono-inoculation; (CL): Simultaneous inoculation of yeast-Concerto and oenococcal-Lalvin; (SL): Sequential inoculation of yeast-Concerto and oenococcal-Lalvin.

retained and/or generated the widest variety of volatile compounds (desirable esters including ethyl acetate and ethyl-(*Z*)-4-hexanoate, and ketones including 2-heptanone). Our results highlighted the feasibility of the sequential inoculation strategy for the development of a pleasant, more complex and aromatic fermented SCG beverage.

CRediT authorship contribution statement

Yunjiao Liu: Methodology, Data curation, data, Formal analysis, Visualization, Writing – original draft, preparation, Writing – review & editing. **Xin Yi Chua:** Data curation, data, Formal analysis, Visualization, Writing – original draft, preparation. **Wenjiang Dong:** Validation, Writing – review & editing, Resources. **Yuyun Lu:** Conceptualization, Methodology, Writing – review & editing, Validation, Supervision. **Shao-Quan Liu:** Conceptualization, Methodology, Writing – review & editing, Validation, Project administration, Supervision, All authors reviewed the results and approved the final version of the manuscript.

Declaration of competing interest

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

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2022.08.002.

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