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Volatile composition of bilberry wines fermented with non-*Saccharomyces* and *Saccharomyces* yeasts in pure, sequential and simultaneous inoculations

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ABSTRACT

Bilberry (*Vaccinium myrtillus* L.) juice was fermented with *Torulaspora delbrueckii* (TD291 and TD70526) and *Schizosaccharomyces pombe* (SP3796 and SP70572) in pure fermentation as well as in sequential and simultaneous inoculations with *Saccharomyces cerevisiae* 1116 (SC1116). Altogether, 56 volatile compounds were identified and semi-quantified with HS-SPME-GC/MS in bilberry products. Yeast fermentation prominently enhanced the aroma complexity of bilberry with a sharp increase in alcohols, esters, aldehydes, and acetals. Compared to *S. cerevisiae, T. delbrueckii* produced less ethanol but more fusel alcohols that potentially enhance "alcohol" and "nail polish" odors in TD70526 and less "fruity" esters in TD291. SP70572 resulted in high productions of undesirable compounds of acetoin and acetaldehyde but a low content of higher alcohols and esters, SP3796 produced a high content of fatty acid ethyl esters and acetoin. In comparison with monoculture of non-*Saccharomyces* yeast, sequential and simultaneous cultures of *S. pombe* and *S. cerevisiae* significantly decreased the content of acetoin while increased the relative level of esters; sequential cultures of *T. delbrueckii* and *S. cerevisiae* remarkably increased the concentration of acetaldehyde; simultaneous inoculations of *S. cerevisiae* with TD70526 and TD291 significantly decreased the content of fusel alcohols and increased the content of esters, respectively. The findings suggested that non-*Saccharomyces* yeasts possess the potential to affect and modulate the aromatic profile of fermented bilberry products. Sequential and simultaneous inoculations with *S. pombe* strains and *S. cerevisiae* as well as simultaneous fermentation using *T. delbrueckii* strains and *S. cerevisiae* are optimal strategies to positively influence the aroma profile of bilberry wines.

1. Introduction

Aroma in fermented products is a quality character arising from a complex combination of volatile compounds, which represent diverse structures, such as alcohols, esters, terpenes, and aldehydes. The aroma-active volatile compounds may contribute individually or synergistically to the aromatic profile of fermented products and play a key role in determining the unique sensory quality of the final products. For instance, alcohols and aldehydes typically contribute to "herbaceous" and "solvent-like" odors, esters to "fruity" aroma, and terpenoid compounds to "floral" character in wines (Cheynier et al., 2010).

Yeast fermentation is a complex metabolic process that converts sugars and amino acids in the fruit must to ethanol, carbon dioxide and an abundance of secondary volatile metabolites. The choice of yeast defines the individuality of fermented products, including color, mouthfeel and aroma complexity, due to the variation in the metabolic characteristics among yeast species and strains. *Saccharomyces cerevisiae* is the "conventional" yeast species extensively used in the wine industry due to high fermentation ability, tolerance to harsh fermentative conditions, and low risk of spoilage reflected as low production of off-flavor compounds (Albergaria and Arneborg, 2016). However, the common metabolic characteristics of the commercially available *Saccharomyces* yeasts have resulted in final wine products of similar chemical composition with low diversity of especially flavor compounds (Albergaria and Arneborg, 2016).

Non-*Saccharomyces* yeasts have recently been applied in winemaking in order to improve the aroma complexity or mouthfeel of wines (Padilla et al., 2016; Sun et al., 2014). Among the non-*Saccharomyces* strains, *Schizosaccharomyces pombe* and *Torulaspora delbrueckii* are the representative microorganisms for their outstanding fermentative performances in terms of higher ethanol producing capability than other non-*Saccharomyces* yeasts (Benito et al., 2016; Ciani and Maccarelli, 1998). Moreover, *S. pombe* could tolerate the hyperosmotic condition caused by high sugar content (Antonio et al., 2016; Domizio et al., 2017) and other hostile environments such as those with low pH and temperatures variation (Suárez-Lepe et al., 2012). Strains of *T.*

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delbrueckii could proliferate even under anaerobic and freezing conditions (Alves-Araújo et al., 2004; Shekhawat et al., 2017). However, these two strains showed significantly different performances in the production of various flavor-active compounds during fermentation. *S. pombe* has been documented to reduce pH by almost complete consumption of malic acid (Domizio et al., 2017), which is among the key contributors to harsh "green apple sourness" and puckering astringency in berries (Laaksonen et al., 2010; Mylona et al., 2016). Compared to *Saccharomyces* species, *S. pombe* strains produce more flavor compounds that are generally considered as undesirable, such as acetaldehyde, acetic acid, acetoin, and sulfides (Benito et al., 2016; Mylona et al., 2016). On the other hand, strains of *T. delbrueckii* have achieved popularity in the wine industry due to the lower production of the aforementioned off-flavor compounds and concomitant higher yields of fruity esters and higher alcohols in comparison to other non-*Saccharomyces* yeasts (Chen et al., 2018; Taillandier et al., 2014). Recently, sequential and simultaneous inoculations of non-*Saccharomyces* yeasts with *S. cerevisiae* cultures have been applied to increase the fermentation efficiency, to reduce or eliminate the negative influence on wine quality caused by non-*Saccharomyces* yeasts, and to enhance the positive impact on aromatic profile through the complex interaction of strains. (Ciani et al., 2010; Lleixà et al., 2016; Sadoudi et al., 2012).

Bilberry (*Vaccinium myrtillus* L.) has gained the interest of the food, nutraceutical and pharmaceutical industries due to its attractive dark-blue color, desirable taste and health-beneficial effects. Furthermore, bilberries have a higher content of esters that contribute typically to "fruity" aroma than the fruits of other *Vaccinium* species do, such as highbush blueberry (*V. corymbosum* L.) and bog blueberry (*V. uliginosum* L.) (Farneti et al., 2017; Hirvi and Honkanen, 1983). Bilberries are typically consumed fresh or frozen, or they are processed into jams or juices. Processing berries into wine and other fermented beverages is commonly considered an effective manner to circumvent the defects of the brief fruiting season and the short shelf life of berries. Moreover, fermentation results in distinctive flavors in its products in contrast to unfermented ones. Nitrogen is among the key elements influencing the growth and metabolism of yeasts during fermentation. The lack of bilberry wine products on the market may be partly due to the slow fermentation of the berries by yeast because of the low level of nitrogen sources in the berry matrices of *Vaccinium* species (Seo et al., 2015; Wang et al., 2016). The use of diverse yeast species, including *S. cerevisiae* and non-*Saccharomyces* yeasts, under the same nitrogen conditions results in significantly different fermentation kinetics. Moreover, sequential and simultaneous inoculations have significant impacts on fermentation rates due to the interactions between microorganisms (Kemsawasd et al., 2015; Lleixà et al., 2016; Taillandier et al., 2014).

Our previous study (Liu et al., 2018) has shown that fermentation with non-*Saccharomyces* yeasts of *T. delbrueckii* and *S. pombe* had a significant impact on the color parameters and anthocyanin composition of bilberry wines. Volatile compounds play a crucial role in the aroma and flavor of juices and wines. No previous research has been published on the influence of these two non-*Saccharomyces* yeast strains on volatile compounds of bilberry fermented products. In the present study, we investigated the impact of fermentation on bilberry juice with pure cultures of *S. pombe* and *T. delbrueckii* on the volatile composition in comparison to *S. cerevisiae*. Additionally, the profiles of volatile compounds of bilberry wines produced with sequential and simultaneous inoculations of non-*Saccharomyces* yeasts and *S. cerevisiae* were studied.

2. Materials and methods

2.1. Bilberry juice preparation

Frozen wild bilberry fruits of Finnish origin were bought from a local supermarket in Turku, Finland. The fruits were kept at −20 °C until processing and analysis. Skinless, seedless and sterile bilberry juices were prepared according to our previous method (Liu et al., 2018). The °Brix and pH values of the final bilberry juice were 20.0 and 3.5, respectively, with the adjustment of sucrose and sodium hydroxide. Afterwards, an aliquot of 80 mL juice was divided into 100 mL a sterile Duran bottle.

2.2. Yeast strains and fermentation procedures

The cultures of *Saccharomyces cerevisiae* 1116 (SC1116) and *Torulaspora delbrueckii* 291 (TD291) were obtained from Lallemand Inc. (Montreal, Canada). *Schizosaccharomyces pombe* 3796 (SP3796), *Schizosaccharomyces pombe* 70572 (SP70572), and *Torulaspora delbrueckii* 70526 (TD70526) were provided by the DSMZ Institute (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany).

The proliferation of *S. cerevisiae* 1116, *T. delbrueckii* 291 and *S. pombe* 3796 in YPD liquid medium (10 g/L yeast extract, 20 g/L peptone and 20 g/L dextrose) and *T. delbrueckii* 70526 and *S. pombe* 70572 in YM liquid medium (3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, and 10 g/L glucose) was conducted as described in our previous study (Liu et al., 2018). To obtain yeast cells, the broth was centrifuged at $4500 \times g$ for 10 min to remove supernatant. Afterwards, the biomass was washed by resuspension in 0.9% sterile sodium chloride solution, followed by centrifugation at $4500 \times g$ for 10 min. The washing was repeated three times, after which the pellet was collected and resuspended in the same medium for fermentation.

Prior to inoculation, the yeast cell population was determined by the spread plate technique. Three types of fermentation were conducted: pure fermentations (PR) by inoculation with a single *S. cerevisiae* or non-*Saccharomyces* yeast strain; sequential fermentation (SQ) by inoculation with a non-*Saccharomyces* yeast as the starter strain to reduce the °Brix to 10.0, followed by inoculation with the *S. cerevisiae* to complete the fermentation; and simultaneous fermentation (SM) by co-inoculation with *S. cerevisiae* and non-*Saccharomyces* yeast at the same time. All the cultures were inoculated when the yeast cell counts reached the level of 10^7 CFU/mL. Blended complex yeast nutrient (Fermaid K™, Lallemand Inc.) was added to the bilberry media at a concentration of 40 mg/100 mL juice at the beginning of fermentation and during the fermentation when the °Brix value was reduced by one third of the original value of bilberry juice. The fermentation temperature was maintained at 25 °C, and all experiments were performed in duplicate. During the fermentation, the weight loss of the fermentation bottles as the release of CO_2 was monitored every two day as the estimation of fermentation kinetics. In sequential fermentation, the °Brix value was measured every two days in the early stage of fermentation, but the monitoring of °Brix was carried out daily when the °Brix value reduced to approximately 11.0, and the frequency of monitoring was again once every two days after the inoculation of *S. cerevisiae*. Fermentation was assumed complete when the °Brix values and the weight remained constant (i.e., < 0.1 g weight loss two days) at two consecutive monitoring time points. After fermentation, all of the bilberry wines were centrifuged at $2800 \times g$ for 10 min to remove yeast cells and the solids. The supernatants were kept at −80 °C until analysis. The bilberry juice, which went through the same "fermentation" procedure as did the bilberry wines for 40 days but without yeast inoculation, was used as the control.

2.3. Measurements of pH, Brix, residual sugar, and ethanol content

The pH values were measured with a pH meter (WTW, Weilheim, Germany) and the °Brix values with a portable Brix meter (Atago Co. Ltd., Tokyo, Japan). Analysis of individual sugars was carried out in duplicate with a gas chromatograph equipped with a flame ionization detector (GC-FID, Shimadzu, Japan, model GC-2010plus) and an SPB-1 column (30 m × 0.25 mm i.d., 0.25 μm, Supelco, Bellefonte, PA) according to the previous method (Liu et al., 2018). Individual sugars

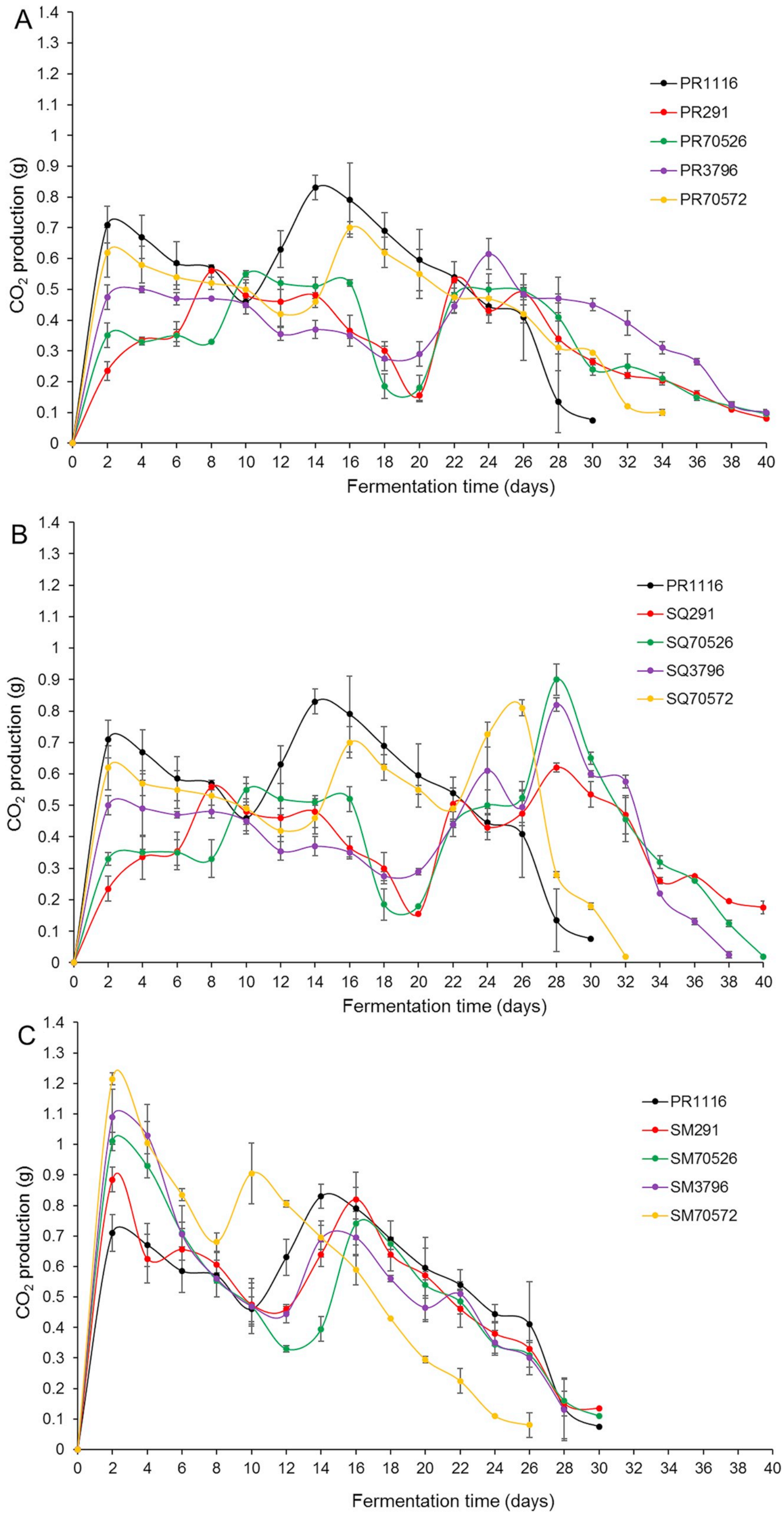

Fig. 1. Fermentation kinetics (explained by CO_2 production every two days) of bilberry wines produced by pure (PR), sequential (SQ) and simultaneous (SM) fermentations with non-*Saccharomyces* yeasts and *Saccharomyces cerevisiae*.

were identified by comparing the retention times with those of the reference compounds. Sorbitol was used as the internal standard to quantify the analytes. The total sugar content was calculated as the sum of concentrations of all individual sugars. Ethanol was measured using a method described in the previous study (Liu et al., 2018) in duplicate with GC-FID (Shimadzu, Japan, model GC-2010plus) equipped with an

Table 1
General parameters of unfermented juice and bilberry wines produced by pure, sequential and simultaneous fermentations with non-*Saccharomyces* yeasts and *Saccharomyces cerevisiae*.

Treatment [a]		pH	Ethanol (%,v/v)	Total sugar [b]
	Juice	3.54 ± 0.01a	–	248.59 ± 5.30d
Pure fermentation	SC1116	3.56 ± 0.01 ab	11.72 ± 0.04g	0.40 ± 0.03a
	TD291	3.57 ± 0.01abc	9.93 ± 0.02a	27.52 ± 1.68c
	TD70526	3.58 ± 0.03abcde	10.39 ± 0.11b	16.53 ± 0.53b
	SP3796	3.60 ± 0.01bcdef	11.21 ± 0.03cde	0.94 ± 0.08a
	SP70572	3.62 ± 0.00def	11.42 ± 0.13ef	0.57 ± 0.05a
Sequential fermentation with SC1116	TD291	3.57 ± 0.00abcd	11.02 ± 0.06c	0.44 ± 0.02a
	TD70526	3.61 ± 0.01cdef	11.13 ± 0.06cd	0.27 ± 0.01a
	SP3796	3.64 ± 0.00efg	11.42 ± 0.01ef	0.36 ± 0.00a
	SP70572	3.63 ± 0.01ef	11.57 ± 0.14 fg	0.52 ± 0.03a
Simultaneous fermentation with SC1116	TD291	3.58 ± 0.03abcde	11.34 ± 0.16de	0.37 ± 0.01a
	TD70526	3.63 ± 0.00 fg	11.42 ± 0.11ef	0.86 ± 0.02a
	SP3796	3.69 ± 0.01h	11.60 ± 0.04 fg	0.40 ± 0.01a
	SP70572	3.68 ± 0.01gh	11.67 ± 0.06g	0.82 ± 0.07a

Results represent the mean ± SD. Values in the same column with different superscript letters are considered significantly different at $P < 0.05$ (ANOVA with Tukey's test).
[a] SC1116, TD291, TD70526, SP3796, and SP70572 are *Saccharomyces cerevisiae* 1116, *Torulaspora delbrueckii* 291, *Torulaspora delbrueckii* 70526, *Schizosaccharomyces pombe* 3796, and *Schizosaccharomyces pombe* 70572, respectively.
[b] Total sugar = sum of individual sugars.

HP-INNOWax column (30 m × 0.25 mm i.d., 0.25 μm, Hewlett-Packard, Avondale, PA). In brief, the oven temperature was started at 40 °C, held for 8 min, raised to 240 °C at a rate of 10 °C/min, and kept for 2 min. Aliquots of 0.2 μL of each sample were injected in the split mode with a split ratio of 1:25. The injector temperature was 220 °C, and the detector temperature was 280 °C. Helium was used as the carrier gas at a flow rate of 1.5 mL/min. Authentic ethanol (> 99.5%) was used as the external standard. A calibration curve ($R^2 > 0.999$) was constructed by analysis of a series of ethanol solutions of concentrations 0, 5, 10, 15 and 20% in ultrapure water.

2.4. Determination of volatile compounds

The volatile compounds in the bilberry products were analyzed in duplicate using headspace solid phase microextraction coupled with analyzed gas chromatography-mass spectrometry (HS-SPME-GC-MS). Two milliliters of each sample and 0.2 g of sodium chloride were placed in a 20 mL glass vial, and 10 μL of 4-methyl-2-pentanol solution (802 μg/mL in methanol) was added as an internal standard. The volatile compounds were extracted from the headspace with a 2 cm DVB/CAR/PDMS fiber (50/30 μm, Supelco, Bellefonte, PA) at 45 °C for 30 min. The fiber was conditioned at 250 °C prior to sample extraction. After the extraction, the SPME fiber was immediately transferred to the injection port of a Trace 1310 gas chromatograph equipped with a TSQ 8000 EVO mass spectrometer (Thermo Fisher Scientific, Waltham, MA) to be thermally desorbed in the splitless mode at 240 °C for 3 min. Two different columns, a DB-WAX polar capillary column (60 m × 0.25 mm i.d. × 0.25 μm film thickness, J&W Scientific, Folsom, CA) and an SPB-624 mid-polarity capillary column (60 m × 0.25 mm i.d. × 1.4 μm film thickness, Supelco, Bellefonte, PA), were used to separate the volatile compounds of the samples. Helium was used as the carrier gas at a flow rate of 1.6 mL/min. The initial column temperature was set at 50 °C and held for 3 min. Afterwards, the temperature was increased to 200 °C at a rate of 5 °C/min and held at 200 °C for 8 min. Mass spectra were detected in electron impact (EI) mode at 70 eV with a scan range from *m/z* 33 to *m/z* 300. The MS transfer line and the ionization source temperatures were 200 and 220 °C, respectively. The RIs of the volatiles were calculated via co-injection with an alkane mixture (C5-C30, Sigma-Aldrich, St. Louis, MO). Volatiles were identified by matching the obtained mass spectra with the standard NIST 08 library and by comparing the retention indices (RI) to those of the compounds reported in the literature and the NIST Webbook (https://webbook.nist.gov/chemistry/). Moreover, the identification of a selected number of volatile compounds was confirmed by comparing the retention indices and mass spectra with those of the authentic reference compounds. Semi-quantification of individual compounds separated with the DB-WAX column was performed by comparing the base peak areas at their respective retention times (Table 2) to the area of the base peak of the internal standard (I.S.) (Beckner Whitener et al., 2015; García-Carpintero et al., 2011), using the following equation:

$$C\ (\mu g/L) = \frac{A_C}{A_{I.S.}} C_{I.S.}(\mu g/L)$$

C: relative concentration of analyte; $C_{I.S.}$: final concentration of internal standard in samples; A_C: peak area of analyte; $A_{I.S.}$: peak area of internal standard.

The semi-quantification analysis did not involve determination or application of any correction factors between individual compounds and the internal standards, and the concertation range for linearity response was also not determined.

2.5. Statistical analysis

The semi-quantitative data were used for statistical comparison and multivariate analysis to compare the samples produced by different treatments. The differences between the means were examined by one-way ANOVA and Tukey's test using SPSS 21.0 (SPSS Inc., Chicago, IL). Statistical significance was set at $P < 0.05$. Multivariate models were created with Unscrambler X software (version 10.5, Camo Inc., Norway). Unsupervised classification, principal component analysis (PCA), was applied to study the sample groupings and correlations among volatile profiles (X-data) of the bilberry wine samples. Partial least squares discriminant analysis (PLS-DA) was used to further study the classification of volatile data according to yeast species or fermentation type.

3. Results and discussion

3.1. Fermentation kinetics

The fermentation kinetics, expressed as the production of CO_2, of the four non-*Saccharomyces* yeasts in pure, sequential and simultaneous fermentations in comparison with the kinetics of the fermentation with pure *S. cerevisiae* 1116 (PR1116) are illustrated in Fig. 1 A, B and C, respectively. The data correspond to the mean values of duplicate fermentations. *S. cerevisiae* produced the highest CO_2 in the early stage of pure fermentation, followed by *S. pombe* and *T. delbrueckii* (Fig. 1 A). The strains of *S. cerevisiae* and *S. pombe* in pure fermentations released the maximum CO_2 on days 2–4. In contrast, the maximum values of CO_2 release in the pure fermentations with *T. delbrueckii* strains were reached on days 8–10. This indicates *S. cerevisiae* and *S. pombe* strains

Table 2

Identification of volatile compounds and their odor descriptor and odor series in unfermented juice and bilberry wines produced by pure, sequential and simultaneous fermentations with non-*Saccharomyces* yeasts and *Saccharomyces cerevisiae*.

No. [a]	Compounds	RT [b]		RI [c]		BP [d]	Formula	Identification [e]	Odor descriptor	Odor series [f]	Reference [g]
		DB-WAX	SPB-624	DB-WAX	SPB-624						
	C6 alcohols										
1	1-Hexanol	17.24		1351		56	$C_6H_{14}O$	MS, RI, STD	Herbaceous, grass, woody	1	1
2	(*Z*)-3-Hexen-1-ol	18.14		1385		67	$C_6H_{12}O$	MS, RI, STD	Green, cut grass	1	2
3	(*E*)-2-Hexen-1-ol	18.70		1405		57	$C_6H_{12}O$	MS, RI	Herbaceous, green,	1	3
	Higher alcohols										
4	1-Propanol	8.58	10.38	1035	610	45	C_3H_8O	MS, RI, STD	Alcohol, ripe fruit	2, 4	1
5	2-Methyl-1-propanol	10.08	12.89	1094	677	43	$C_4H_{10}O$	MS, RI, STD	Alcohol, nail polish	4	1
6	1-Butanol	11.40		1142		56	$C_4H_{10}O$	MS, RI	Medicinal, alcohol	4	4
7	2-Methyl-1-butanol	13.15	17.37	1204	789	57	$C_5H_{12}O$	MS, RI, STD	Nail polish, malt	4	5
8	3-Methyl-1-butanol	13.22	17.23	1206	786	55	$C_5H_{12}O$	MS, RI, STD	Nail polish, alcohol	4	6
9	4-Methyl-1-pentanol	16.18		1312		56	$C_6H_{14}O$	MS, RI	Almond, toasted	6	7
10	3-Methyl-1-pentanol	16.54		1325		56	$C_6H_{14}O$	MS, RI	Pungent, alcohol, green	1, 4	8
11	3-Ethoxy-1-propanol	17.97		1379		59	$C_5H_{12}O_2$	MS, RI	Fruity	2	8
12	1-Heptanol	20.00		1456		70	$C_7H_{16}O$	MS, RI	Oily	5	2
13	2-Ethyl-1-hexanol	20.88	28.08	1490	1070	57	$C_8H_{18}O$	MS, RI	Rose	3	9
14	1-Octanol	22.64		1560		56	$C_8H_{18}O$	MS, RI	Jasmine, lemon	3	8
15	*Threo*-2,3-butanediol	23.09		1560		45	$C_4H_{10}O_2$	MS, RI	Fruity	2	8
16	2-Phenylethanol	30.93	31.40	1925	1169	91	$C_8H_{10}O$	MS, RI	Rose, honey	3	1
	Esters										
17	Ethyl acetate	5.56	11.50	886	740	43	$C_4H_8O_2$	MS, RI, STD	Pineapple, fruity, pungent	2, 4	1
18	Ethyl 3-methylbutyrate	9.28		1063		45	$C_7H_{14}O_2$	MS, RI	Fruity, apple	2	10, 11
19	4-Methyl-2-pentyl acetate	10.27		1101		43	$C_8H_{16}O_2$	MS, RI	Sweet, fruity, banana	2	12
20	Isoamyl acetate	10.70	21.98	1117	904	43	$C_7H_{14}O_2$	MS, RI, STD	Banana, fruity, sweet	2	8
21	Ethyl hexanoate	13.88	26.39	1230	1022	88	$C_8H_{16}O_2$	MS, RI	Fruity, green apple, banana, brandy, wine-like	2	1
22	Isopentyl 3-methylbutyrate	15.63		1292		70	$C_{10}H_{20}O_2$	MS, RI	Fruity	2	13
23	Ethyl heptanoate	16.72		1332		88	$C_9H_{18}O_2$	MS, RI	Fruity	2	11, 13
24	Ethyl lactate	16.99		1342		45	$C_5H_{10}O_3$	MS, RI	Fruity, buttery	2, 5	1
25	Methyl 2-hydroxy-3-methylbutanoate	18.60		1402		73	$C_6H_{12}O_3$	MS, RI			
26	Ethyl caprylate	19.47	31.65	1435	1177	88	$C_{10}H_{20}O_2$	MS, RI	Sweet, fruity	2, 3	2
27	2,6,8-Trimethyl-4-nonanol	22.53		1556		69	$C_{12}H_{26}O$	MS, RI			
28	Methyl decanoate	23.61		1599		43	$C_{11}H_{22}O_2$	MS, RI	Winey, fruity, flora	2	14
29	Ethyl caprate	24.63		1600		88	$C_{12}H_{24}O_2$	MS, RI	Fruity	2	2
30	Diethyl succinate	25.51		1680		101	$C_8H_{14}O_4$	MS, RI	Fruity, melon	2	1
31	Ethyl 9-decenoate	25.88		1691		88	$C_{12}H_{22}O_2$	MS, RI	Fruity	2	14
32	Ethyl 4-hydroxybutanoate	28.46		1810		74	$C_6H_{12}O_3$	MS, RI	Caramel, flower	7	11, 13
33	Phenethyl acetate	28.87		1829		104	$C_{10}H_{12}O_2$	MS, RI	Flowery	3	1
34	Ethyl dodecanoate	29.30		1848		88	$C_{14}H_{28}O_2$	MS, RI	Brandy, fruity, grape	2	1
	Monoterpenes										
35	Linalool	22.32		1547		71	$C_{10}H_{18}O$	MS, RI	Citrus, floral, sweet, grape-like	2, 3	1
36	α-Terpineol	26.14		1707		59	$C_{10}H_{18}O$	MS, RI	Lilac, floral, sweet	3	1
37	β-Citronellol	27.56		1769		45	$C_{10}H_{20}O$	MS, RI	Rosy	3	8
	Ketones										
38	2-Pentanone	7.17	15.00	974	731	43	$C_5H_{10}O$	MS, RI	Ether, pungent, fruit	2, 4	9, 15
39	4-Methyl-2-pentanone	7.82		1005		43	$C_6H_{12}O$	MS, RI	Strawberry, sweet, varnish	2	13
40	4,6-Dimethyl-2-heptanone	14.23		1242		43	$C_9H_{18}O$	MS, RI			
41	Acetoin	15.48		1287		45	C4H8O2	MS, RI	Buttery, fatty	5	8
42	6-Methyl-5-hepten-2-one	16.88		1338		43	$C_8H_{14}O$	MS, RI, STD	Herbaceous, green, cabbage	1	9
43	2,6,8-Trimethyl-4-nonanone	18.49	32.31	1397	1197	57	$C_{12}H_{24}O$	MS, RI			
	Aldehydes										
44	Acetaldehyde	3.93	5.86	703	< 500	44	C_2H_4O	MS, RI	Ripe apple, Pungent	2, 4	8
45	3-Methylbutanal	6.06	13.57	917	695	44	$C_5H_{10}O$	MS, RI	Malt	4	9, 16
46	Hexanal	9.66		1077		44	$C_6H_{12}O$	MS, RI	Fatty, herbaceous, green	1, 5	3
47	(*E*)-2-Hexenal	13.53		1217		55	$C_6H_{10}O$	MS, RI	Grassy, pungent	1	17
48	Nonanal	18.40	30.08	1394	1151	57	$C_9H_{18}O$	MS, RI, STD	Green, slightly pungent	1	7
49	Benzaldehyde	21.99		1534		105	C_7H_6O	MS, RI	Roasted, almond	3, 6	3
	Acetals										
50	1-Ethoxy-1-methoxyethane	5.05	13.00	843	680	59	$C_5H_{12}O_2$	MS, RI			
51	1,1-Diethoxyethane	5.62	15.49	891	743	45	$C_6H_{14}O_2$	MS, RI	Cake, fruity	2	3
52	2,4,5-Trimethyl-1,3-dioxolane	6.60	15.67	943	747	45	$C_6H_{12}O_2$	MS, RI			
53	2,4-Dimethyl-1,3-dioxane	7.30	16.84	1002	775	45	$C_6H_{12}O_2$	MS, RI			
54	1-(1-Ethoxyethoxy)-pentane	10.24	24.95	1100	982	73	$C_9H_{20}O_2$	MS, RI			

(*continued on next page*)

Table 2 (*continued*)

No. [a]	Compounds	RT [b]		RI [c]		BP [d]	Formula	Identification [e]	Odor descriptor	Odor series [f]	Reference [g]
		DB-WAX	SPB-624	DB-WAX	SPB-624						
Benzenes											
55	1,3,5-Trimethylbenzene	14.30	25.69	1243	1002	105	C_9H_{12}	MS, RI			
56	1,3-Di-tert-butylbenzene	19.32	32.99	1429	1208	175	$C_{14}H_{22}$	MS, RI			

[a] Number of volatile compounds detected by DB-WAX.
[b] Retention time of volatile compounds detected by DB-WAX and SPB-624 columns.
[c] Retention indices of volatile compounds on DB-WAX and SPB-624 columns.
[d] Base peak of mass spectrum.
[e] Identification method of volatile compounds, MS: mass spectrum, RI: retention index, STD: standard.
[f] Odor series 1: herbaceous, 2: fruity, 3: floral, 4: chemical, 5: fatty, 6: roasted.
[g] Odor descriptor and odor series references. 1. (Peinado et al., 2004), 2. (Sánchez-Palomo et al., 2010), 3. (Franco et al., 2004), 4. (Li et al., 2008), 5. (Rapp and Versini, 1995), 6. (Siebert et al., 2005), 7. (Cai et al., 2014), 8. (Peinado et al., 2006), 9. (Comuzzo et al., 2006), 10. (Culleré et al., 2004), 11. (http://www.flavornet.org/flavornet.html), 12. (http://www.thegoodscentscompany.com), 13. (http://www.vcf-online.nl/VcfCompounds.cfm), 14. (Englezos et al., 2016), 15. (Viljanen et al., 2014), 16. (Zhu et al., 2018).

had stronger fermentation capabilities and reached the maximum cell population earlier than *T. delbrueckii* strains did. The results are in agreement with previous studies (Belda et al., 2015; Domizio et al., 2017). The high level of CO_2 production was maintained for 6–8 days and then reduced remarkably, probably due to the depletion of nutrients in the fermented matrix. As expected, the second addition of nitrogen sources enhanced the metabolism of yeasts. Afterwards, the values of CO_2 release decreased continuously until the completion of fermentation. It is worth noting that the fermentation of the bilberry wines with pure non-*Saccharomyces* yeasts was 4–10 days longer than the fermentation with SC1116 (30 days) (Fig. 1A). The slower fermentation kinetics of non-*Saccharomyces* yeasts could be explained by their higher nitrogen requirements for growth (Lleixà et al., 2016). Supplementation of nitrogen sources in *Vaccinium* species musts has been previously reported to shorten their fermentation durations (Seo et al., 2015; Wang et al., 2016). For these reasons, nitrogen source was added into bilberry juice twice during the fermentation of bilberry wine. The participation of *S. cerevisiae* in sequential fermentation increased the CO_2 production dramatically in the later stage (Fig. 1 B). Compared to the fermentation with non-Saccharomyces yeast alone, the simultaneous inoculations of non-*Saccharomyces* yeast and *S. cerevisiae* remarkably expedited the fermentation rates, and all the fermentations were completed within 30 days. The rapid reduction of CO_2 release in the later stage of simultaneous fermentations may result from the competitive or antagonistic effect of these two types of yeast in nitrogen sources (Comitini et al., 2011; Sadoudi et al., 2012) (Fig. 1 C).

3.2. General parameters

Table 1 presents the general parameters of the bilberry wines and the control juice. Compared to the juice, fermentation involving *S. pombe* strains significantly increased the pH value. Moreover, the increase correlated positively with the duration of participation of *S. cerevisiae* 1116. However, the fermentations with *S. cerevisiae* and *T. delbrueckii* strains showed less of an impact on the pH values. Fermentation with non-*Saccharomyces* yeasts yielded a lower content of ethanol than fermentation with *S. cerevisiae* did due to greater accumulation of yeast biomass or byproducts of non-*Saccharomyces* yeasts after the consumption of sugars (Ciani et al., 2016; Ciani and Maccarelli, 1998). Furthermore, the lowest concentration of ethanol in pure fermentation with *T. delbrueckii* strains was also ascribed to the incomplete consumption of sugars. Compared with pure fermentation with non-*Saccharomyces* yeast, the inoculation of *S. cerevisiae* in sequential and simultaneous fermentations with *T. delbrueckii* led to the complete consumption of sugars accompanied by a significant increase in ethanol levels. However, the significant increase was only detected in simultaneous fermentations involving *S. pombe*.

3.3. Analysis of volatile compounds

A total of 56 volatile compounds were detected by HS-SPME-GC-MS using the DB-WAX column in the bilberry wines and the control juice (Table 2, Supplementary Figs. 1 and 2). The volatile compounds included sixteen alcohols, eighteen esters, three monoterpenes, six ketones, six aldehydes, five acetals, and two benzenes. A second column, SPB-624, was applied to detect potential volatile compounds that may not have been separated with the first column DB-WAX. Moreover, the use of the second column assisted in identification of the volatile compounds through comparison of retention indices to the literature and RI databases.

Semi-quantitative analysis of the 56 volatile compounds detected by the DB-WAX column was performed in order to investigate the impact of fermentation processes on the composition of volatile compounds. The results are summarized in Table 3. The quantification was based on direct comparison of base peak areas of individual compounds with that of the internal standard in the total ion chromatograms, which is a commonly applied practice for semi-quantification of volatile compounds (Beckner Whitener et al., 2015; D'Agostino et al., 2015; Nicolle et al., 2018; Ricci et al., 2018). Due to the lack of authentic reference compounds, no correction factors were determined or applied to compensate for the difference in behaviors between individual compounds and the internal standard during the SPME-GC-MS analysis process. Therefore, the levels of the volatile compounds measured in this study were used only for comparison of different samples and not taken as absolute concentrations of these compounds in the samples.

3.3.1. Difference in volatile composition between bilberry wines and control juice as impact of yeast fermentation

The fermented samples and the control juice showed significant differences in volatile profile. The total contents of each group of volatiles in bilberry wines were significantly higher than those in the control juice except those of the monoterpenes and ketones (Table 3). Among the 56 volatiles detected, 27 compounds, mainly belonging to the groups of higher alcohols and esters, were detected only in the wine samples. Seven of the 56 compounds, mainly ketones and aldehydes, were not detected after fermentation (Table 3, Supplementary Fig. 1).

The PLS-DA model was used to compare the profiles of volatile compounds (X-data, n = 56) in the control juice and the wine samples produced by pure fermentation (Y-data, n = 4) (Fig. 2) and to identify compounds contributing to the differences among the samples. In the model with three validated factors ($R^2 = 0.905$; validated $R^2 = 0.873$), the control juice was clearly separated from the pure fermentation samples on the first factor by the volatile variables detected only in the juice and the fermented samples. Acetoin and acetaldehyde, which are key compounds possessed negative effects on the organoleptic

Table 3
Semi-quantification of volatile compounds (μg/L) in unfermented juice and bilberry wines produced by pure, sequential and simultaneous fermentations with non-*Saccharomyces* yeasts and *Saccharomyces cerevisiae*.

No.	Compounds	Juice	Pure fermentation				
			PR1116	PR291	PR70526	PR3796	PR70572
C6 alcohols							
1	1-Hexanol	6.20 ± 0.4a	18.85 ± 1.20bcde	18.48 ± 1.25bcde	15.88 ± 0.83b	19.73 ± 1.17de	26.17 ± 0.73g
2	(*Z*)-3-Hexen-1-ol	2.60 ± 0.07a	14.27 ± 0.85bcd	13.17 ± 1.27bc	14.40 ± 1.28bcd	13.81 ± 0.49bc	17.47 ± 0.43ef
3	(*E*)-2-Hexen-1-ol	1.78 ± 0.12	ND	ND	ND	ND	ND
	Total	6.20 ± 0.40a	33.12 ± 2.04bc	31.65 ± 2.51bc	30.28 ± 2.08b	33.54 ± 1.64bc	43.63 ± 0.89ef
Higher alcohols							
4	1-Propanol	ND	301.8 ± 50.02b	190.05 ± 21.06a	646.86 ± 45.72f	415.91 ± 23.20cd	157.53 ± 5.56a
5	2-Methyl-1-propanol	184.25 ± 12.92a	3718.76 ± 388.42cd	4495.76 ± 521.09def	10309.07 ± 509.59i	3577.84 ± 326.60c	2562.07 ± 122.35b
6	1-Butanol	ND	46.31 ± 2.76abc	68.1 ± 3.66cde	82.38 ± 4.30e	36.72 ± 2.56 ab	31.17 ± 0.24a
7	2-Methyl-1-butanol	124.08 ± 5.90a	7805.62 ± 477.52cd	8470.91 ± 791.42de	11101.53 ± 1688.06g	6356.32 ± 395.06cd	3577.29 ± 1324.92b
8	3-Methyl-1-butanol	616.30 ± 23.59a	32437.41 ± 2311.98ef	31204.51 ± 2166.03cd	51013.03 ± 3405.22f	26679.37 ± 1272.13bc	14201.44 ± 648.76b
9	4-Methyl-1-pentanol	ND	26.26 ± 1.74e	9.79 ± 0.62bc	15.39 ± 3.44d	12.13 ± 0.95cd	2.74 ± 0.11a
10	3-Methyl-1-pentanol	ND	40.92 ± 6.3def	44.52 ± 7.11ef	25.84 ± 6.64bc	23.92 ± 2.08b	1.96 ± 0.32a
11	3-Ethoxy-1-propanol	ND	2.66 ± 0.33a	16.42 ± 6.07a	205.08 ± 13.57b	10.51 ± 0.59a	1.33 ± 0.39a
12	1-Heptanol	8.71 ± 3.32abc	13.28 ± 0.78cdef	13.68 ± 2.35def	6.51 ± 0.33a	16.14 ± 2.88ef	8.13 ± 0.62 ab
13	2-Ethyl-1-hexanol	571.62 ± 29.63i	148.53 ± 8.90 fg	167.75 ± 9.64gh	87.65 ± 5.64bcd	52.58 ± 4.10a	79.55 ± 4.72abc
14	1-Octanol	10.67 ± 1.51e	4.58 ± 0.49a	5.07 ± 0.42 ab	4.86 ± 0.36a	5.54 ± 0.49 ab	5.92 ± 0.53abc
15	*Threo*-2,3-butanediol	ND	365.29 ± 85.14 ab	155.28 ± 73.25a	297.65 ± 100.07 ab	572.32 ± 188.81 ab	420.94 ± 47.70 ab
16	2-Phenylethanol	41.83 ± 4.54a	4670.34 ± 460.45e	8641.20 ± 522.41gh	10191.18 ± 1035.19i	4385.74 ± 292.46de	599.50 ± 40.78 ab
	Total	1557.47 ± 44.37a	49581.80 ± 3584.07def	53483 ± 3627.20fgh	83987.00 ± 6632.52i	42145.00 ± 1926.11cd	21649.60 ± 1931.88b
Esters							
17	Ethyl acetate	1348.02 ± 30.17a	11257.59 ± 1016.89c	7836.29 ± 809.29b	11426.16 ± 275.41cd	7629.03 ± 481.34b	8882.52 ± 340.92b
18	Ethyl 3-methylbutyrate	ND	10.48 ± 1.03a	4.08 ± 0.45a	3.49 ± 0.84a	5.38 ± 0.97a	1.04 ± 0.39a
19	4-Methyl-2-pentyl acetate	9.77 ± 0.64c	5.75 ± 0.55b	4.07 ± 0.14 ab	4.62 ± 0.30 ab	4.51 ± 0.40 ab	1.92 ± 2.01a
20	Isoamyl acetate	16.67 ± 1.24a	262.47 ± 25.22de	143.95 ± 8.9abcd	457.64 ± 193.02f	163.97 ± 42.25abcd	44.37 ± 1.87 ab
21	Ethyl hexanoate	ND	108.98 ± 6.83a	73.94 ± 7.78a	71.06 ± 22.19a	390.96 ± 66.54d	103.38 ± 4.64a
22	Isopentyl 3-methylbutyrate	ND	0.20 ± 0.09a	0.19 ± 0.09a	0.16 ± 0.06a	0.25 ± 0.19a	0.52 ± 0.34a
23	Ethyl heptanoate	ND	2.35 ± 0.42 ab	1.94 ± 0.14a	1.90 ± 0.48a	14.58 ± 2.32e	3.66 ± 0.47 ab
24	Ethyl lactate	ND	139.81 ± 9.05e	193.25 ± 13.03f	117.27 ± 14.12d	126.93 ± 5.00de	80.09 ± 2.84 ab
25	Methyl 2-hydroxy-3-methylbutanoate	26.68 ± 1.03f	4.50 ± 0.23d	3.64 ± 0.22bcd	1.33 ± 0.17a	3.47 ± 0.02bc	3.7 ± 0.37bcd
26	Ethyl caprylate	ND	129.44 ± 42.41bcd	67.29 ± 4.39abc	29.44 ± 10.93a	681.55 ± 15.88f	56.9 ± 6.08abc
27	2,6,8-Trimethyl-4-nonanol	44.19 ± 0.94e	16.21 ± 0.22 ab	17.20 ± 0.51bc	18.19 ± 0.60c	21.35 ± 0.48d	15.84 ± 0.44 ab
28	Methyl decanoate	ND	2.88 ± 0.97 ab	1.58 ± 0.32 ab	0.81 ± 0.22a	24.65 ± 4.80d	1.83 ± 0.65 ab
29	Ethyl caprate	ND	245.58 ± 115.11bc	132.95 ± 18.30 ab	32.62 ± 7.01a	783.4 ± 205.52e	58.24 ± 19.49a
30	Diethyl succinate	ND	34.74 ± 3.57e	21.95 ± 1.35cd	18.47 ± 4.65bc	14.65 ± 2.13abc	6.49 ± 0.70a
31	Ethyl 9-decenoate	ND	18.83 ± 3.49cd	20.48 ± 5.35cde	3.12 ± 1.02a	29.12 ± 2.13e	4.16 ± 7.06 ab
32	Ethyl 4-hydroxybutanoate	ND	45.50 ± 9.68de	38.70 ± 7.27cde	24.24 ± 2.08abc	33.91 ± 2.75bcd	7.56 ± 1.52a
33	Phenethyl acetate	ND	67.31 ± 5.73b	129.84 ± 6.50c	270.91 ± 39.04d	54.00 ± 3.82b	3.43 ± 0.32a
34	Ethyl dodecanoate	ND	45.53 ± 16.07b	18.13 ± 1.57 ab	6.64 ± 0.93a	161.31 ± 44.99c	5.23 ± 1.57a
	Total	1445.32 ± 30.62a	12398.20 ± 1176.86c	8709.49 ± 813.37b	12488.10 ± 216.45c	10143.00 ± 355.12b	9280.85 ± 312.87b
Monoterpenes							
35	Linalool	53.82 ± 8.66e	21.66 ± 2.27 ab	20.73 ± 3.46a	32.56 ± 4.45cd	26.27 ± 4.11abc	36.78 ± 2.14d
36	*α*-Terpineol	35.01 ± 1.85c	8.23 ± 0.53 ab	8.64 ± 0.87 ab	9.11 ± 0.50b	8.57 ± 0.14 ab	7.28 ± 0.85 ab
37	*β*-Citronellol	ND	5.53 ± 0.56de	3.34 ± 0.35bc	5.54 ± 0.90de	3.10 ± 0.14bc	0.75 ± 1.14a
	Total	88.83 ± 9.89d	35.43 ± 2.47 ab	32.71 ± 3.79a	47.20 ± 5.77c	37.93 ± 3.91abc	44.80 ± 2.40bc
	Ketones						
38	2-Pentanone	128.09 ± 6.10	ND	ND	ND	ND	ND
39	4-Methyl-2-pentanone	287.94 ± 6.07	ND	ND	ND	ND	ND
40	4,6-Dimethyl-2-heptanone	34.92 ± 0.80	ND	ND	ND	ND	ND
41	Acetoin	ND	22.94 ± 2.13a	6.57 ± 1.24a	4.54 ± 1.57a	165.16 ± 35.84b	278.34 ± 36.86c
42	6-Methyl-5-hepten-2-one	10.30 ± 1.83	ND	ND	ND	ND	ND
43	2,6,8-Trimethyl-4-nonanone	68.97 ± 2.5f	29.95 ± 2.23abc	27.17 ± 1.89 ab	31.32 ± 2.25bcd	34.75 ± 1.71cde	25.90 ± 1.00 ab

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Table 3 (*continued*)

No.	Compounds	Juice	Pure fermentation				
			PR1116	PR291	PR70526	PR3796	PR70572
	Total	530.22 ± 8.83e	52.89 ± 1.47 ab	33.74 ± 2.21 ab	35.87 ± 0.91 ab	199.92 ± 35.24c	304.24 ± 36.65d
Aldehydes							
44	Acetaldehyde	ND	1591.99 ± 256.26cd	1032.64 ± 62.85 ab	930.59 ± 126.60a	1344.58 ± 276.99abcd	3375.42 ± 452.78f
45	3-Methylbutanal	ND	31.37 ± 2.26e	16.7 ± 3.43bc	17.32 ± 2.95bcd	32.64 ± 5.31e	3.75 ± 1.46a
46	Hexanal	44.63 ± 5.84	ND	ND	ND	ND	ND
47	(*E*)-2-Hexenal	2.14 ± 0.46	ND	ND	ND	ND	ND
48	Nonanal	5.07 ± 1.20 ab	8.40 ± 0.48c	6.64 ± 1.01abc	5.54 ± 0.96abc	6.97 ± 1.69abc	5.77 ± 1.53abc
49	Benzaldehyde	19.47 ± 5.06bc	1.71 ± 0.26a	15.05 ± 3.05b	4.73 ± 0.33a	6.18 ± 3.60a	38.60 ± 4.73d
	Total	69.16 ± 1.36a	1633.47 ± 256.36de	1071.03 ± 67.27bc	958.18 ± 123.2b	1390.37 ± 275.2bcde	3423.56 ± 448.09g
Acetals							
50	1-Ethoxy-1-methoxyethane	ND	684.38 ± 136.37f	177.21 ± 8.97abc	162.37 ± 37.44abc	254.18 ± 61.41bcd	131.08 ± 19.79 ab
51	1,1-Diethoxyethane	ND	2188.96 ± 488.34e	514.89 ± 32.79abc	540.42 ± 112.87abc	896.86 ± 217.02bcd	469.72 ± 62.45 ab
52	2,4,5-Trimethyl-1,3-dioxolane	ND	2990.49 ± 446.80f	666.18 ± 22.34abcd	359.90 ± 74.12abc	2089.86 ± 275.75e	206.76 ± 20.35 ab
53	2,4-Dimethyl-1,3-dioxane	ND	174.81 ± 27.12e	46.96 ± 4.26 ab	29.27 ± 7.36a	114.69 ± 21.50c	37.61 ± 6.73a
54	1-(1-Ethoxyethoxy) pentane	ND	209.07 ± 54.88c	52.62 ± 3.26 ab	90.37 ± 19.05b	61.78 ± 17.34 ab	13.84 ± 2.32a
	Total	ND	6247.70 ± 1128.16e	1457.87 ± 57.55 ab	1182.33 ± 249.73 ab	3417.36 ± 591.93c	859.00 ± 71.503a
Benzenes							
55	1,3,5- Trimethylbenzene	70.75 ± 2.59a	226.27 ± 10.61bcd	202.47 ± 16.50b	245.08 ± 7.49cde	239.90 ± 4.83cd	226.24 ± 10.75bcd
56	1,3-Di-tert-butylbenzene	119.82 ± 13.31ef	136.13 ± 24.91f	92.34 ± 10.73bcdef	44.71 ± 4.56 ab	62.69 ± 14.32abcde	34.17 ± 3.46a
	Total	190.57 ± 11.18a	362.40 ± 34.44f	294.80 ± 15.12bcd	289.8 ± 11.95bcd	302.59 ± 16.50bcde	260.41 ± 11.41b

No.	Sequential fermentation with SC1116				Simultaneous fermentation with SC1116			
	SQ291	SQ70526	SQ3796	SQ70572	SM291	SM70526	SM3796	SM70572
C6 alcohols								
1	17.29 ± 0.65bcd	16.64 ± 1.67bc	21.35 ± 0.46ef	30.44 ± 0.83h	20.34 ± 0.88ef	19.17 ± 1.37cd	22.75 ± 2.26f	32.09 ± 1.21h
2	12.44 ± 0.54b	13.64 ± 0.30bc	14.77 ± 0.53cd	17.62 ± 0.23ef	15.05 ± 0.36cd	14.79 ± 0.80cd	16.12 ± 1.46de	19.08 ± 0.82f
3	ND	ND	ND	ND	ND	ND	ND	ND
	29.72 ± 1.16b	30.28 ± 1.92b	36.12 ± 0.99cd	48.06 ± 0.72 fg	35.39 ± 1.22cd	33.96 ± 2.17bc	38.87 ± 3.69de	51.18 ± 1.98g
Higher alcohols								
4	310.00 ± 5.52b	817.40 ± 42.47g	495.29 ± 23.26e	181.95 ± 4.64a	360.86 ± 16.42bc	459.66 ± 31.33de	440.70 ± 36.98de	190.09 ± 4.65a
5	5157.24 ± 87.21fgh	10750.08 ± 176.49i	4582.36 ± 404.15efg	4844.22 ± 86.40efg	4124.30 ± 27.79cde	5639.94 ± 490.30h	4688.07 ± 490.21efg	5391.96 ± 119.53gh
6	73.37 ± 2.37de	112.87 ± 3.98f	114.92 ± 29.44f	46.08 ± 0.88abc	48.83 ± 2.31abc	58.19 ± 4.20bcd	49.22 ± 4.54abc	28.15 ± 0.55a
7	9474.43 ± 397.16defg	11924.56 ± 783.97g	9363.38 ± 259.64defg	11508.37 ± 383.47gh	8204.47 ± 224.68cde	10337.40 ± 763.87f	8939.27 ± 767.09def	10904.75 ± 1948.09fgh
8	35426.12 ± 868.47ef	53839.72 ± 1824.33f	33615.17 ± 963.49ef	22332.89 ± 892.87c	33920.97 ± 1044.89ef	37561.01 ± 3634.49f	35056.65 ± 3894.04ef	24388.44 ± 721.3c
9	12.33 ± 0.41cd	11.58 ± 0.33cd	14.88 ± 0.36d	4.79 ± 0.14a	23.62 ± 0.36e	33.27 ± 2.38f	26.61 ± 3.33e	6.45 ± 0.44 ab
10	36.45 ± 1.07de	25.94 ± 0.99bc	38.13 ± 0.84de	7.28 ± 0.18a	34.71 ± 1.07cd	49.16 ± 1.74f	36.64 ± 5.35de	9.11 ± 0.19a
11	15.45 ± 0.70a	225.50 ± 5.00b	11.88 ± 0.97a	1.16 ± 0.16a	4.93 ± 0.35a	12.88 ± 0.17a	5.90 ± 0.72a	1.12 ± 0.09a
12	13.93 ± 0.83def	8.14 ± 0.94 ab	14.73 ± 0.53def	11.06 ± 0.82abcd	15.51 ± 2.47def	11.99 ± 0.69bcde	17.18 ± 3.22f	13.94 ± 0.94def
13	165.38 ± 13.8gh	65.81 ± 0.75 ab	74.94 ± 3.36abc	53.57 ± 2.80a	187.21 ± 28.55h	127.05 ± 7.02ef	106.36 ± 8.99cde	115.70 ± 7.10def
14	4.82 ± 0.62a	4.58 ± 0.22a	7.34 ± 0.24d	5.39 ± 0.35 ab	5.16 ± 0.83 ab	6.05 ± 0.08abc	6.57 ± 0.60cd	7.27 ± 0.35d
15	365.70 ± 164.26 ab	237.51 ± 73.09 ab	1304.70 ± 424.79c	710.66 ± 53.45b	312.10 ± 55.28 ab	422.28 ± 163.13 ab	505.61 ± 217.31 ab	609.93 ± 305.94 ab
16	8085.00 ± 188.50g	9593.63 ± 391.58hi	4871.78 ± 257.05e	1373.12 ± 66.04b	4814.83 ± 267.95e	5921.78 ± 146.38f	3453.75 ± 378.18cd	2644.30 ± 104.10c
	59140.20 ± 923.32gh	87617.30 ± 2653.03i	54509.50 ± 1321.32fgh	41080.50 ± 1401.20c	52057.50 ± 1236.51 fg	60640.70 ± 4926.02h	53332.50 ± 5487.69fgh	44311.20 ± 2174.30cde
Esters								
17	13507.42 ± 742.07d	11134.55 ± 808.13c	17686.62 ± 1380.88f	14232.42 ± 822.12e	16674.57 ± 531.99f	11217.77 ± 585.98c	10910.26 ± 1025.91c	7933.82 ± 284.39b
18	5.00 ± 0.39a	6.88 ± 0.32a	7.67 ± 0.23a	1.78 ± 1.14a	56.62 ± 55.15b	7.66 ± 0.94a	6.48 ± 4.31a	3.96 ± 0.30a
19	3.21 ± 2.48 ab	5.46 ± 0.29b	3.16 ± 2.04 ab	2.64 ± 1.56 ab	5.46 ± 0.29b	4.97 ± 0.15 ab	3.86 ± 0.16 ab	3.22 ± 1.92 ab
20	245.88 ± 34.4cde	1173.23 ± 68.51h	630.68 ± 50.11g	191.64 ± 9.82bcd	205.35 ± 58.41bcd	668.47 ± 57.18g	396.87 ± 25.49ef	89.46 ± 3.05abc
21	72.70 ± 14.49a	115.25 ± 16.36a	463.32 ± 38.13e	231.93 ± 9.68b	90.19 ± 6.32a	236.45 ± 5.73b	356.19 ± 37.2cd	295.31 ± 11.61bc
22	0.45 ± 0.32a	0.23 ± 0.1a	0.43 ± 0.52a	0.37 ± 0.25a	6.78 ± 7.69b	0.22 ± 0.05b	0.23 ± 0.04a	0.25 ± 0.19a

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Table 3 (*continued*)

No.	Sequential fermentation with SC1116				Simultaneous fermentation with SC1116			
	SQ291	SQ70526	SQ3796	SQ70572	SM291	SM70526	SM3796	SM70572
23	1.66 ± 0.20a	5.05 ± 1.96bc	14.16 ± 2.28e	8.52 ± 0.14d	1.75 ± 0.10a	3.9 ± 0.17 ab	7.49 ± 1.14cd	5.29 ± 0.24bc
24	178.09 ± 15.87f	106.4 ± 4.57cd	111.55 ± 3.20d	63.37 ± 2.54a	143.83 ± 7.22e	109.72 ± 9.35d	85.29 ± 7.73bc	61.92 ± 1.92a
25	3.23 ± 0.13b	1.11 ± 0.21a	3.64 ± 0.27bcd	3.61 ± 0.31bcd	4.37 ± 0.22cd	3.11 ± 0.25b	3.55 ± 0.49bcd	4.02 ± 0.37bcd
26	54.28 ± 15.51 ab	148.18 ± 90.57cd	594.70 ± 66.93f	85.50 ± 10.26abcd	89.26 ± 3.21abcd	162.49 ± 3.39d	459.73 ± 33.05e	170.96 ± 9.79d
27	16.37 ± 0.65 ab	16.03 ± 0.71 ab	21.34 ± 1.09d	14.76 ± 0.29a	15.28 ± 0.21a	21.12 ± 1.26d	17.18 ± 0.68bc	18.45 ± 0.29c
28	0.51 ± 0.12a	1.75 ± 0.58 ab	8.35 ± 1.72cd	0.86 ± 0.19a	2.04 ± 0.12 ab	2.69 ± 0.21 ab	10.34 ± 1.02c	5.33 ± 1.76bc
29	50.34 ± 23.00a	83.26 ± 42.64 ab	350.55 ± 80.04cd	17.87 ± 5.56a	135.18 ± 2.01 ab	162.3 ± 6.46 ab	432.65 ± 39.92d	177.37 ± 31.77abc
30	29.95 ± 1.24de	30.49 ± 3.24de	32.97 ± 3.03e	9.97 ± 1.16 ab	34.59 ± 1.67e	17.81 ± 15.3bc	17.07 ± 1.37abc	11.25 ± 0.78abc
31	12.44 ± 5.44bc	12.84 ± 4.06bc	46.46 ± 1.37f	6.60 ± 0.83 ab	12.34 ± 1.69bc	23.86 ± 3.48de	23.96 ± 0.94de	18.98 ± 1.82cd
32	52.65 ± 7.63de	14.60 ± 1.00 ab	19.32 ± 2.29abc	5.76 ± 0.49a	22.39 ± 21.25abc	23.51 ± 4.85abc	53.91 ± 6.67e	8.30 ± 1.45a
33	128.4 ± 10.40c	276.04 ± 29.46d	74.47 ± 9.21b	6.87 ± 0.17a	66.19 ± 1.25b	126.73 ± 7.43c	54.39 ± 4.49b	8.53 ± 0.55a
34	7.89 ± 4.53a	8.10 ± 3.49a	49.19 ± 13.52b	3.01 ± 0.55a	19.81 ± 8.99 ab	20.91 ± 1.47 ab	31.12 ± 2.81 ab	15.69 ± 1.12 ab
	13890.50 ± 690.86cd	13139.40 ± 1028.94c	20618.60 ± 1162.72f	15425.70 ± 336.88d	17586.00 ± 541.99e	12856.00 ± 654.3c	12547.00 ± 561.03c	8832.13 ± 327.29b
Monoterpenes								
35	20.55 ± 0.75a	26.14 ± 1.98abc	22.72 ± 3.44 ab	30.21 ± 0.64bcd	21.02 ± 0.63a	20.98 ± 1.02a	21.12 ± 2.28a	30.37 ± 1.55bcd
36	8.14 ± 0.27 ab	8.31 ± 0.76 ab	8.97 ± 0.87 ab	6.91 ± 0.92a	8.21 ± 0.49 ab	8.68 ± 0.67 ab	7.25 ± 0.81 ab	8.03 ± 0.40 ab
37	4.42 ± 0.37cd	6.13 ± 0.78def	6.96 ± 1.35ef	2.05 ± 0.19 ab	5.50 ± 0.28de	7.74 ± 0.46f	4.55 ± 0.38cd	1.64 ± 0.25 ab
	33.11 ± 1.20a	40.57 ± 2.93abc	38.65 ± 4.26abc	39.17 ± 1.36abc	34.72 ± 1.2 ab	37.40 ± 1.55abc	32.91 ± 2.96a	40.04 ± 1.49abc
38	ND	ND	ND	ND	ND	ND	ND	ND
39	ND	ND	ND	ND	ND	ND	ND	ND
40	ND	ND	ND	ND	ND	ND	ND	ND
41	6.24 ± 0.92a	4.65 ± 1.13a	7.82 ± 0.44a	4.14 ± 0.23a	20.03 ± 2.93a	5.36 ± 0.36a	11.77 ± 1.99a	36.83 ± 3.57a
42	ND	ND	ND	ND	ND	ND	ND	ND
43	28.82 ± 1.19 ab	31.3 ± 4.25bcd	37.83 ± 2.03e	24.50 ± 2.52a	26.88 ± 2.78 ab	37.04 ± 2.25de	31.29 ± 1.45bcd	31.45 ± 1.43bcd
	35.06 ± 2.09 ab	35.95 ± 5.04 ab	45.65 ± 2.16 ab	28.64 ± 2.65a	46.91 ± 4.45 ab	42.40 ± 2.05 ab	43.05 ± 3.33 ab	68.28 ± 4.95b
Aldehydes								
44	1546.64 ± 86.17cd	1487.06 ± 151.85bcd	1817.42 ± 44.22d	3005.19 ± 27.11ef	1130.01 ± 115.18abc	903.27 ± 57.79a	1537.42 ± 79.87cd	2524.90 ± 211.75e
45	25.82 ± 5.76cde	57.34 ± 8.62f	24.45 ± 3.18cde	21.23 ± 9.61bcde	29.69 ± 3.92de	45.34 ± 2.05f	27.46 ± 4.72cde	10.24 ± 0.93 ab
46	ND	ND	ND	ND	ND	ND	ND	ND
47	ND	ND	ND	ND	ND	ND	ND	ND
48	6.90 ± 2.01abc	6.04 ± 0.94abc	7.73 ± 1.12abc	4.68 ± 1.25a	8.23 ± 1.74c	8.02 ± 0.29bc	8.23 ± 1.29c	6.51 ± 0.17abc
49	3.18 ± 0.72a	2.29 ± 0.75a	2.78 ± 0.31a	2.81 ± 0.41a	1.92 ± 0.24a	6.05 ± 0.75a	4.62 ± 0.55a	24.62 ± 1.40c
	1582.54 ± 94.25de	1552.73 ± 157.93cde	1852.37 ± 42.85e	3033.91 ± 36.71 fg	1169.84 ± 116.74bcd	962.68 ± 59.03b	1577.73 ± 85.18de	2566.27 ± 213.01f
Acetals								
50	286.82 ± 8.49cd	362.34 ± 45.64d	167.13 ± 23.57abc	109.83 ± 8.49a	554.11 ± 58.63e	220.16 ± 9.19abc	165.7 ± 4.85abc	128.04 ± 4.08 ab
51	914.89 ± 27.16cd	1205.89 ± 165.21d	598.85 ± 88.72abc	391.08 ± 21.65a	1813.37 ± 137.72e	799.19 ± 103.56abcd	607.05 ± 53.26abc	496.54 ± 36.39abc
52	702.00 ± 59.78bcd	407.50 ± 108.44abcd	872.38 ± 218.15d	183.53 ± 58.24a	2138.05 ± 290.62e	563.95 ± 117.34abcd	774.78 ± 220.82cd	198.10 ± 44.60 ab
53	55.10 ± 1.39 ab	51.38 ± 7.90 ab	69.61 ± 3.81b	55.19 ± 1.00 ab	145.61 ± 6.46d	35.93 ± 3.42a	51.97 ± 4.40 ab	29.69 ± 1.59a
54	96.90 ± 4.69b	202.24 ± 28.06c	54.13 ± 8.53 ab	11.87 ± 7.22a	171.00 ± 13.77c	90.25 ± 8.73b	48.36 ± 4.93 ab	26.29 ± 1.38a
	2055.71 ± 25.48b	2229.34 ± 298.11b	1762.1 ± 332.15 ab	751.50 ± 68.22a	4822.14 ± 423.46d	1709.47 ± 239.26 ab	1647.86 ± 248.82 ab	878.66 ± 84.25a
Benzenes								
55	230.19 ± 5.12bcd	254.75 ± 18.49de	255.84 ± 21.12de	231.34 ± 13.35bcd	210.43 ± 5.78bc	273.88 ± 18.7e	235.48 ± 8.57cd	255.04 ± 16.16de
56	108.13 ± 14.92cdef	80.04 ± 67.74abcdef	62.95 ± 5.87abcde	42.92 ± 7.65 ab	111.79 ± 14.24def	51.71 ± 4.41abc	65.03 ± 12.21abcde	59.78 ± 18.37abcd
	338.32 ± 13.47de	334.79 ± 60.78cde	318.79 ± 25.54bcde	274.26 ± 13.11bc	322.22 ± 10.64bcde	325.58 ± 18.25cde	300.51 ± 16.13bcde	314.82 ± 24.93bcde

Results represent the mean ± SD. Values in the same row with different letters are considered significantly different at $P < 0.05$ (ANOVA with Tukey's test). The concentrations of volatile compounds were determined by semi-quantitation.
PR: pure fermentation, SQ: sequential fermentation, SM: simultaneous fermentation.
ND: not detected.

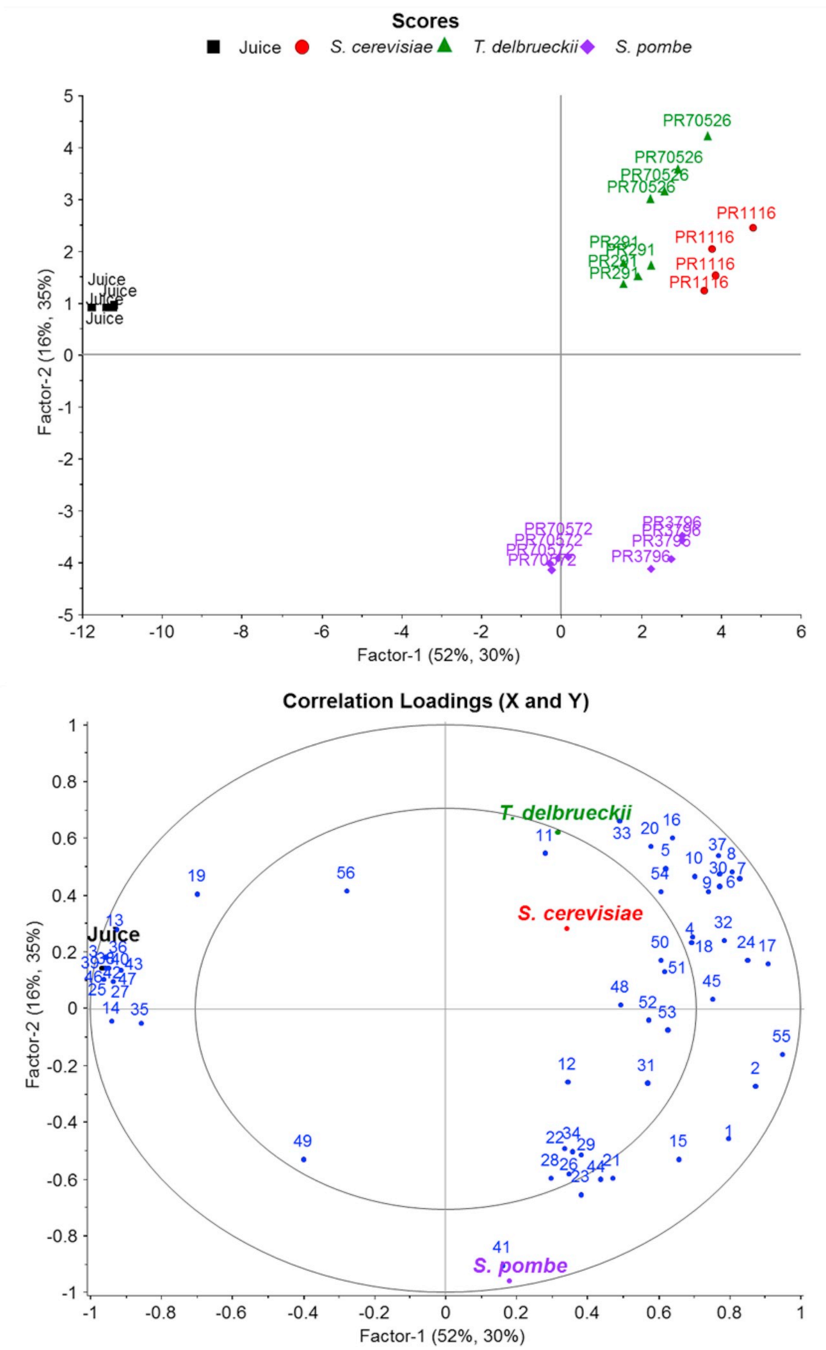

Fig. 2. PLS-DA model using contents of volatiles as X-data (n = 56) to explain the differences between unfermented juice and pure yeast fermented samples (Y-data; n = 4). The variable numbers in the correlation loadings plot refer to Table 2.

properties of wine, were formed during yeast fermentation (Tables 2 and 3). The content of higher alcohols increased significantly after yeast fermentation, with approximately 14–54 times higher values than that in the control juice. However, the contents of two individual higher alcohols of 2-ethyl-1-hexanol and 1-octanol showed a significant decrease. These two compounds typical contributed "floral" aroma (Tables 2 and 3). The reduction in these two alcohols was previously suggested as the utilization for esterification with fatty acids to form esters in lychee wines production with *S. cerevisiae* (Chen and Liu, 2014). The content of esters in bilberry wines was 6–9 times higher than that in the unfermented juice, the fact mainly resulted from the high level of ethyl acetate in the wine samples and the formation of the ten fatty acid ethyl esters (18, 21, 23, 24, 26, and 29–34) during yeast fermentation (Table 3).

3.3.2. Impact of yeast strains and fermentation types on the volatile composition of bilberry wine

To further investigate the impact of different yeasts and fermentation types on the volatile composition of bilberry wine and to detect their differences, PCA models were constructed with the semi-quantitative data obtained from the bilberry wine samples (Fig. 3). The first three principal components (PCs) explained 64% of the total variance. The unsupervised classification model with three principal components showed the influence of different yeast species and strains, being the stronger contributors to the variance in the volatile composition (X-data, n = 49) compared to the fermentation type. The bilberry wines produced by *S. pombe* 70572 in pure (PR70572), sequential (SQ70572) and simultaneous (SM70572) fermentations were located on the negative part of PC1 and were clearly separated from other bilberry wine

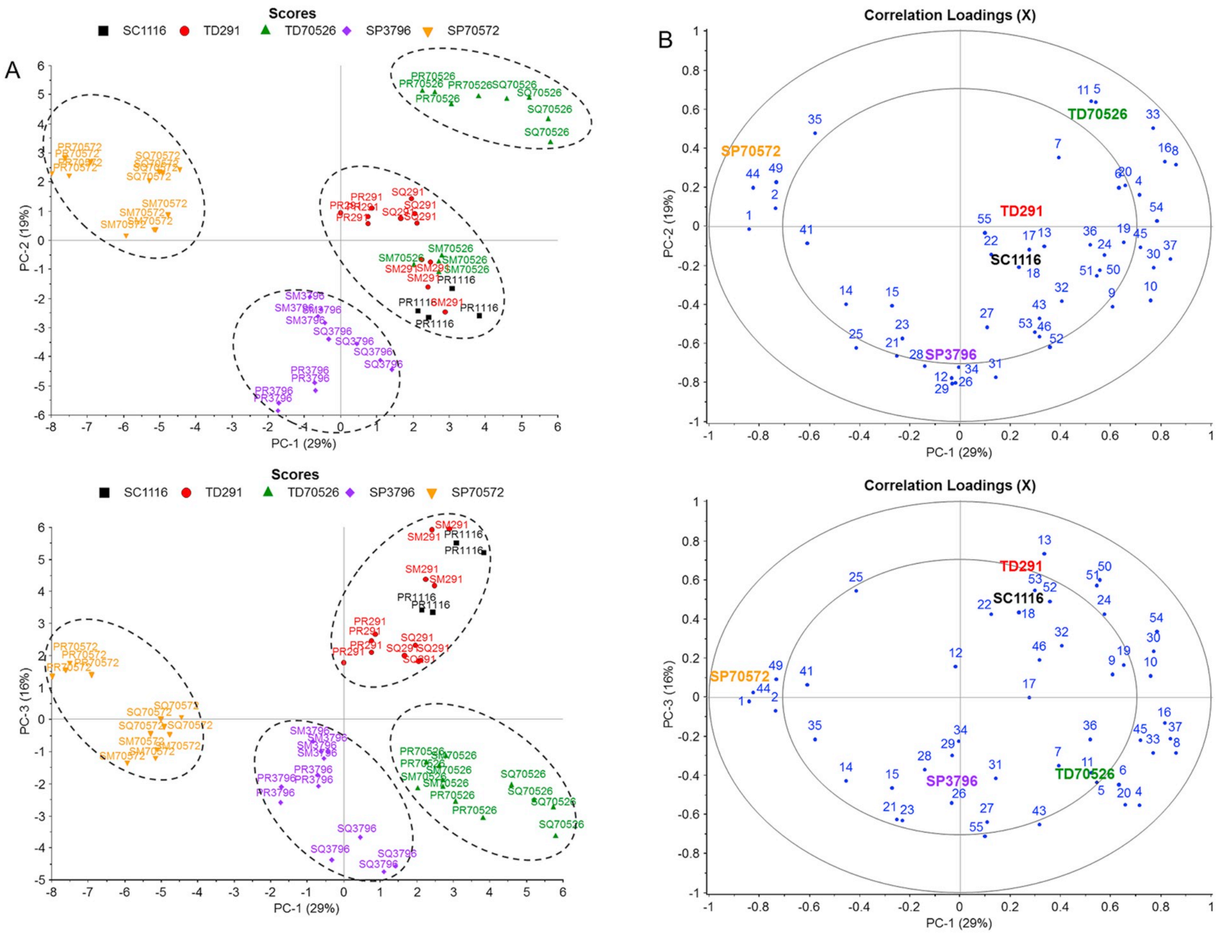

Fig. 3. Principal component analysis (PCA) score plots and correlation loadings plots of the first three principal components based on the data of volatile compounds (49 X-variables) produced by fermentation with non-*Saccharomyces* yeasts and *Saccharomyces cerevisiae* (52 samples). **A.** components 1 and 2; **B.** components 1 and 3. The variable numbers in the correlation loadings plots refer to Table 2.

samples. They were characterized by high contents of C6 alcohols (compounds 1 and 2), acetaldehyde and benzaldehyde, indicating that *S. pombe* 70572 wines may have heavier notes of undesirable "herbaceous", "pungent" and "roasted" odors in comparison to bilberry wines produced with other yeast strains (Table 2, Table 3, Fig. 3). The fermentations involving *S. pombe* 3796 were positioned on the negative sides of PC2 and PC3, with a high concentration of fatty acid ethyl esters, including ethyl heptanoate, ethyl hexanoate, ethyl caprylate, 2,6,8-trimethyl-4-nonanol, ethyl caprate, ethyl 9-decenoate, and ethyl dodecanoate. These ethyl esters may play a positive role in the enhancement of the "fruity" flavor of the bilberry wine. Simultaneous fermentation (SM70526) with *T. delbrueckii* 70526 and *S. cerevisiae* were separated from the pure (PR70526) and sequential fermentation (SQ70526) due to the significant lower production of 1-propanol, 2-methyl-1-propanol, 1-butanol, 2-methyl-1-butanol, 3-methyl-1-butanol, and 3-ethoxy-1-propanol, which are the main contributors to "alcohol" and "nail polish" odors. Meanwhile, PC3 separated the fermentation products of *T. delbrueckii* 291 from the bilberry wines produced by *S. pombe* species through higher production of acetal (51–54), 2-ethyl-1-hexanol and ethyl lactate in the former samples.

3.3.2.1. Yeast strains. Based on the difference between yeast species in the PCA model (Fig. 3), fermentations with *T. delbrueckii* 291 cannot be distinguished clearly from the pure fermentation with *S. cerevisiae*. In order to further investigate the key variables contributing to the differences that were influenced by yeast species but not by fermentation types, a PLS-DA model was established with three validated factors (pure fermentations with different yeast species, $R^2 = 0.946$; validated $R^2 = 0.910$) using the 49 compounds found only in the fermented samples (Fig. 4).

The bilberry wines fermented by *T. delbrueckii* species were located in the upper left quadrant of the loadings plot (Fig. 4), being more abundant with higher alcohols, including 2-methyl-1-propanol, 1-butanol, 2-methyl-1-butanol, 3-methyl-1-butanol, and 3-ethoxy-1-propanol, compared to the bilberry wines produced with the *S. pombe* strains. Similar results were also reported in durian wine fermentation with *T. delbrueckii* (Lu et al., 2016). Among these compounds, 3-methyl-1-butanol, 2-methyl-1-butanol and 2-methyl-1-propanol had the highest relative concentration, together accounting for approximately 90% of the total higher alcohols in bilberry wines (Table 3). The semi-quantitative data suggested that the total content of higher alcohols in the PR70526 wine was approximately 2 and 4 times higher than the levels in the samples of PR3796 and PR70572, respectively (Table 3). Some of the higher alcohols, for example, 1-propanol, 2-methyl-1-propanol, 1-butanol, 2-methyl-1-butanol, 3-methyl-1-butanol, and 3-methyl-1-pentanol, are known to possess "alcohol" or "nail polish" aroma character (Table 2). The contribution of these higher alcohols to the aroma profile of wines is complex, involving both positive and negative

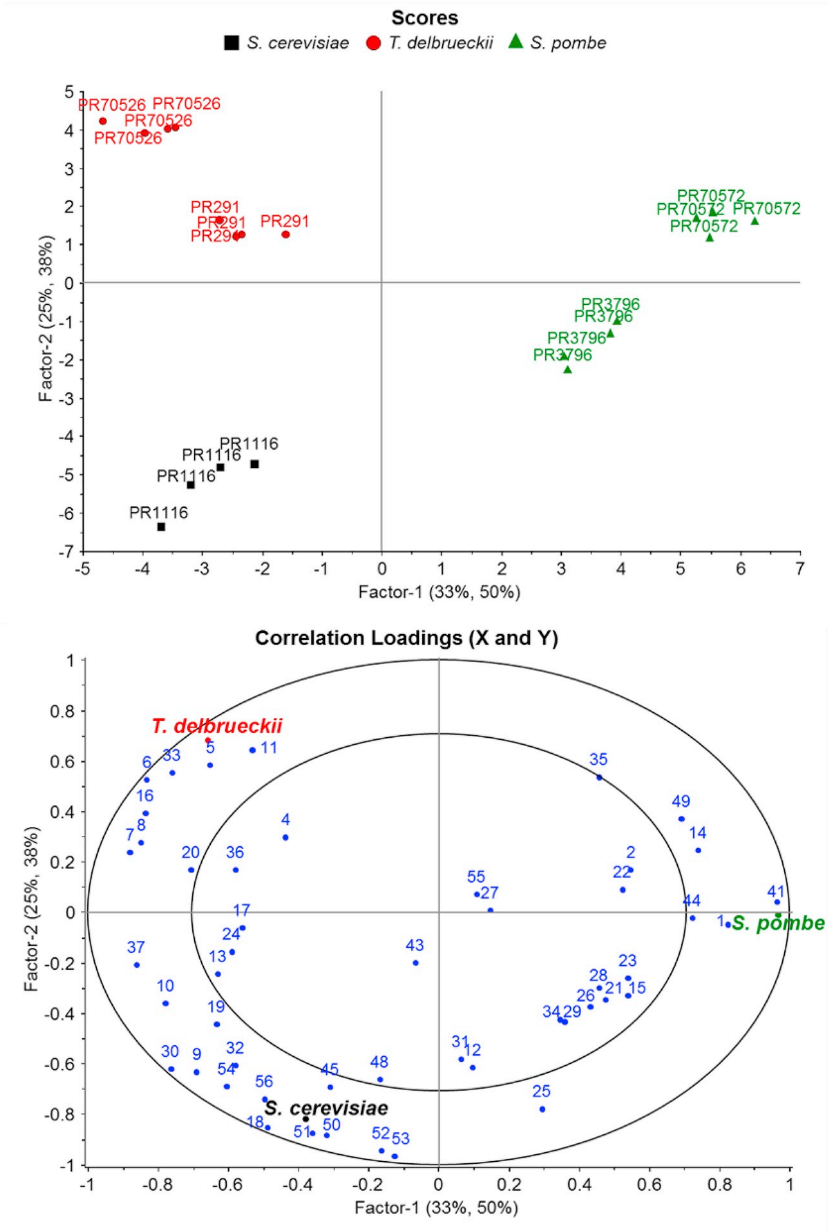

Fig. 4. PLS-DA model using contents of volatiles as X-data (n = 49) to explain the differences between yeasts (Y-data. n = 3) in pure fermentation samples. The variable numbers in the correlation loadings plots refer to Table 2.

effects. A total concentration of these fusel alcohols below 350 mg/L is reported to improve the aroma complexity of wines, whereas a higher content is considered as a defective factor for wines (Rapp and Mandery, 1986). Non-*Saccharomyces* yeasts, except *T. delbrueckii* 70526, yielded less ethyl acetate than *S. cerevisiae* did; ethyl acetate is an important compound responsible for aroma deterioration (Englezos et al., 2018). Among the pure fermentation products, PR70572 had the highest concentration of linalool but the lowest content of *β*-citronellol (Table 3, Fig. 4). Generally, *S. pombe* strains produced more off-flavor compounds of acetoin and acetaldehyde than *T. delbrueckii* did in bilberry wine production; the result is in agreement with a previous study of fermented red wine with the same species of non-*Saccharomyces* yeasts (Loira et al., 2015). Acetaldehyde was the most abundant aldehyde in bilberry wines accounting for more than 95% of total aldehydes. A high concentration of the compound has been suggested to contribute to the "pungent" odor in wines (Peinado et al., 2006).

Bilberry wine produced with *S. cerevisiae* 1116 alone was separated by factors 1 and 2 from *T. delbrueckii* and *S. pombe* wines due to the higher levels of acetals (50–54, Table 3, Fig. 4), the difference being approximately 7-fold for fermentation products obtained with *S. pombe* 70572. Most of the acetals detected in this study have been documented as odorless compounds, except 1,1-diethoxyethane, which has been shown to contribute to the "fruity" aroma of wines (Etievant and Maarse, 1991). 1,1-Diethoxyethane is formed from the reaction between acetaldehyde and ethanol; 2,4,5-trimethyl-1,3-dioxolane and 2,4-dimethyl-1,3-dioxane are produced from the integration of acetaldehyde with glycerol; and 1-ethoxy-1-methoxyethane and 1-(1-ethoxyethoxy) pentane are synthesized by acetaldehyde and corresponding higher alcohols (Cheynier et al., 2010). Interestingly, the concentrations of these acetals did not have clear correlations with the contents of their precursors (no significant correlation at the $p < 0.01$ level), but the maximum values of these acetals were all found in the fermented samples produced by pure fermentation with *S. cerevisiae* 1116. This suggested that the *S. cerevisiae* strains probably possess

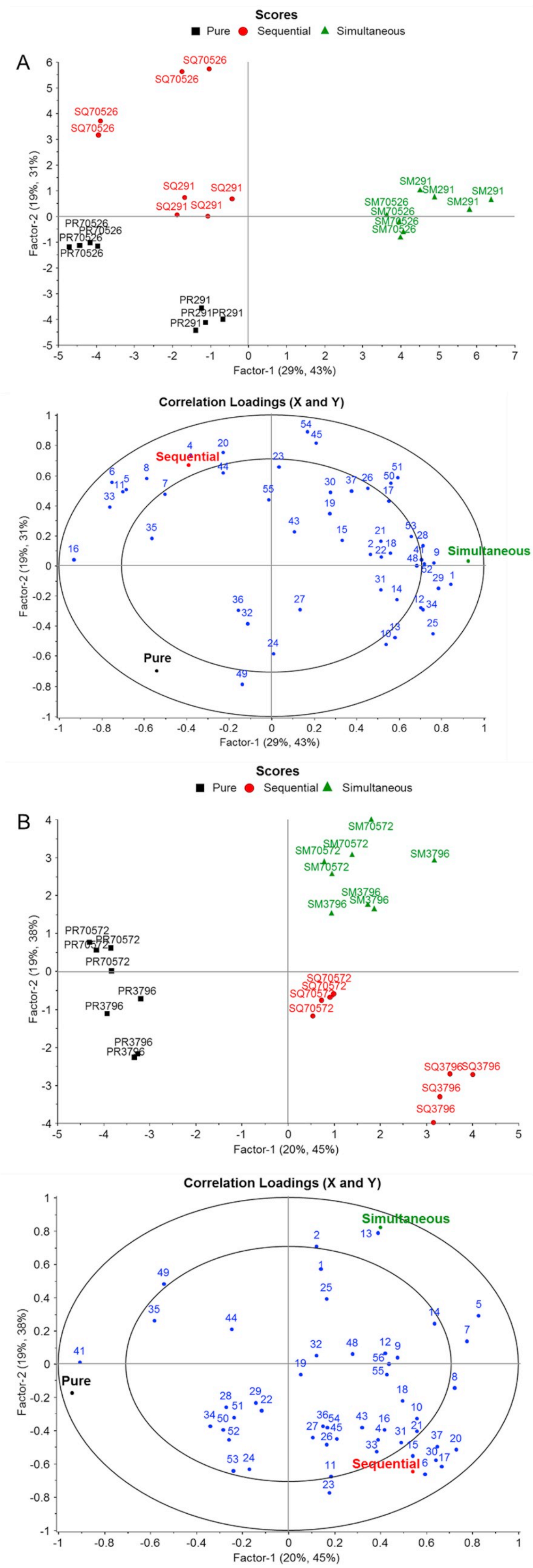

Fig. 5. PLS-DA model using contents of volatiles as X-data (n = 49) to explain the differences between three fermentation types (Y-data. n = 3) in **A.** *T. delbrueckii* and **B.** *S. pombe* samples.

specific metabolic pathways influencing the biosynthesis of acetals.

3.3.2.2. Fermentation types. In the scores plots in PCA (Fig. 3), the bilberry wines fermented with sequential and simultaneous cultures tend to be located closer to the PR1116 sample. However, the bilberry wines produced by non-*Saccharomyces* yeasts with sequential or simultaneous inoculation of *S. cerevisiae* 1116 are not clearly separated from the corresponding products of pure fermentation. Therefore, the impact of fermentation type (pure, sequential and simultaneous) on the composition of volatiles (X-data, n = 49) was further examined with PLS-DA models created for both *T. delbrueckii* (five validated factors, R^2 = 0.958; validated R^2 = 0.909) and *S. pombe* (three validated factors, R^2 = 0.943; validated R^2 = 0.885) (Fig. 5A and B).

Generally, both sequential and simultaneous fermentations contributed to the increase in esters, in comparison with the pure fermentation with non-*Saccharomyces* yeast in the present study (Table 3). This might have been because the levels of esters produced by *S. cerevisiae* were generally higher than those produced by non-*Saccharomyces* yeasts. Sequential and simultaneous inoculations of *S. cerevisiae* with non-*Saccharomyces* yeasts thereby increased the formation of esters. The same phenomenon was also detected previously in lychee and grape wines (Albertin et al., 2017; Loira et al., 2015; Ramírez et al., 2016). The sample of SQ3796 had the highest content of esters among all bilberry wine products, which was approximately 2-fold higher than that of PR3796. The total content of esters correlated positively with the duration of participation of *S. cerevisiae* 1116 in *T. delbrueckii* 291 wines; in contrast, the correlation was negative in *S. pombe* wines. Sequential inoculation significantly increased the concentration of acetaldehyde but showed no impact on the total content of higher alcohols in *T. delbrueckii* samples. While simultaneous fermentation of SC1116/TD70526 significantly decreased the content of higher alcohols. Sequential and simultaneous fermentations with *S. pombe* and *S. cerevisiae* significantly decreased the total content of ketones but increased the contents of higher alcohols and esters. Additionally, sequential fermentation did not influence the content of aldehydes in *S. pombe* wines or the concentration of monoterpenes and benzenes in bilberry wines fermented with both species of non-*Saccharomyces* yeast.

In the PLS-DA models, the sequential and simultaneous fermentations were characterized by more variables compared to the pure fermentations. Interestingly, some of the higher alcohol variables (compounds 4, 6–8), often characterized by the "chemical" odor, and two acetate esters of isoamyl acetate and acetate phenethyl, known as contributing to "fruity" and "floral" notes, respectively, correlated with sequential fermentation for both yeasts. At the same time, 1-hexanol and 2-ethyl-1-hexanol correlated with the simultaneous fermentations.

Higher production of 1-hexanol, 4-methyl-1-pentanol, 1-heptanol, methyl 2-hydroxy-3-methylbutanoate, methyl decanoate, ethyl caprate, ethyl dodecanoate, 2,4,5-trimethyl-1,3-dioxolane, and 2,4-dimethyl-1,3-dioxane distinguished the bilberry wines fermented with a simultaneous inoculation of *T. delbrueckii* strains from the others, as shown in Fig. 5A. In the case of *S. pombe*, acetoin was the key compound separateing the pure fermentation with *S. pombe* strains from the corresponding sequential and simultaneous fermentations (Table 3, Fig. 5B). The inoculation of *S. cerevisiae* 1116 in sequential and simultaneous fermentations with *S. pombe* strains remarkably inhibited the production of this compound (Table 3). Moreover, the first two factors distinguished the sequential fermentations from other types of inoculation by increased release of 1-butanol, *threo*-2,3-butanediol, ethyl acetate, isoamyl acetate, diethyl succinate, and *β*-citronellol (Table 3, Fig. 5B). Most of these compounds potentially contributed to a "fruity" flavor in the fermented products.

4. Conclusion

This is the first study investigating the impact of non-*Saccharomyces* yeasts (*T. delbrueckii* and *S. pombe*) and *S. cerevisiae*, applied in pure, sequential and simultaneous fermentations, on the volatile composition of bilberry wines. All fermentations resulted in sharp increase in the concentrations of most of volatiles, especially those of higher alcohols, esters, aldehydes, and acetals.

Among these non-*Saccharomyces* yeasts, pure culture of *S. pombe* or *T. delbrueckii* strains showed interesting features. In comparison with *S. cerevisiae*, *T. delbrueckii* yeasts generally resulted in low production of ethanol and undesirable compounds of acetoin and acetaldehyde. However, high release of fusel alcohols and low yield of esters were also detected in TD70526 and TD291, respectively. On the contrary, *S. pombe* strains generally increased the production of acetoin and acetaldehyde. SP3796 and SP70572 were also characterized by high yield of "fruity" ethyl esters and low production of fusel alcohols, respectively.

Sequential or simultaneous inoculation of non-*Saccharomyces* yeast with *S. cerevisiae* could significantly weaken the undesirable impacts on flavor caused by pure fermentation with non-*Saccharomyces* yeasts while keep their positive features. Sequential and simultaneous fermentations with *S. pombe* strains and *S. cerevisiae* as well as simultaneous inoculation using *T. delbrueckii* strains and *S. cerevisiae* contributed positively to the aromatic profile of bilberry wines based on the results in the present study. However, the impact of these fermentation strategies on the overall sensory quality of bilberry wine should be further demonstrated with sensory evaluation.

This study provided important information on the effects of yeasts, including *Saccharomyces* and non-*Saccharomyces* yeasts, on the volatile composition of bilberry wines. Further studies are still required to investigate the effects of fermentation with non-*Saccharomyces* yeasts on sensory properties of bilberry wines to support the product development of novel bilberry wines and beverages industry.

Declarations of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2018.12.015.

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