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The Impact of Indigenous Non-*Saccharomyces* Yeasts Inoculated Fermentations on 'Semillon' Icewine

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Abstract: The emerging low acidity in icewine grapes is becoming a major problem in producing quality icewine. Using non-*Saccharomyces cerevisiae* yeasts in fermentation can improve wine's organoleptic characteristics and aromatic quality. This study evaluated two indigenous non-*Saccharomyces cerevisiae* yeasts, *Lachancea thermotolerans* (LT-2) and *Torulaspora delbrueckii* (TD-3), for their ability to improve the acidity and quality of 'Semillon' icewine. Five different inoculation schemes were implemented, including a single inoculation of *S. cerevisiae* (SC), *L. thermotolerans* (LT), and *T. delbrueckii* (TD); the sequential inoculation of *L. thermotolerans*, followed by *S. cerevisiae* after 6 days (L-S); and the sequential inoculation of *L. thermotolerans*, followed by *T. delbrueckii* after 6 days (L-D). The results showed that, during sequential fermentation (L-S and L-D), the presence of *S. cerevisiae* or *T. delbrueckii* slightly restrained the growth of *L. thermotolerans*. Single or sequential inoculation with *L. thermotolerans* and *T. delbrueckii* significantly reduced the amount of volatile acidity and increased the glycerol content. Furthermore, fermentations involving *L. thermotolerans* produced relevant amounts of lactic acid (2.04–2.2 g/L) without excessive deacidification of the icewines. Additionally, sequential fermentations increased the concentration of terpenes, C_{13}-norisoprenoid compounds, and phenethyl compounds. A sensory analysis also revealed that sequentially fermented icewines (L-S and L-D) had more fruity and floral odors and aroma intensity. This study highlights the potential application of *L. thermotolerans* and *T. delbrueckii* in sequential fermentation to improve the icewine quality.

Keywords: *Lachancea thermotolerans*; *Torulaspora delbrueckii*; non-*Saccharomyces cerevisiae* yeasts; sequential fermentation; icewine; volatile compounds

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1. Introduction

Icewine is a type of dessert wine with a golden color and unique aroma similar to citrus fruit, floral, or honey and is very popular among consumers [1]. Icewine is made from naturally frozen grapes (<−8 °C). These grapes are picked, pressed, and fermented at low temperatures [2]. Generally, icewine has a high sugar content and requires high acidity to balance its taste to avoid cloying. Additionally, high-quality icewine needs a complex and typical aroma to support its sensory needs. Due to the harsh winemaking conditions of icewine, it can only be produced in a few regions, hence being known as "liquid gold" [3]. China has become an important icewine production country. Qi Lian is a typical icewine production region in Northwest China with a suitable climate for icewine making [3]. *Vitis. Vinifera* L. cv. Semillon is the typical cultivar used in making icewine, because its berries have relatively thick skins, and the vines are of cold resistance in the Qi Lian Region. However, the 'Semillon' grapes grown in the Qi Lian region have insufficient flavor compounds and natural acidity. This causes serious challenges during the making of 'Semillon' icewine.

In recent years, there has been an increasing interest in using indigenous non-*Saccharomyces* yeasts with organoleptic properties and flavor typicality [4,5]. Suitable icewine yeasts do not

only generate more desired aromatic compounds but also withstand the harsh fermentation conditions, such as high sugar concentration (above 35 °Brix), low temperature (15–18 °C), and high SO_2 and ethanol toxicity during alcoholic fermentation as compared to table wine yeasts [6].

Among the non-*Saccharomyces* yeasts, *Torulaspora delbrueckii* (TD) is the most popular example of these yeasts used in wine production due to its high purity of fermentation products and low production of acetaldehyde, acetic acid, acetoin, and ethyl acetate [7]. In addition, it can increase the content of volatile compounds with impact odorants [8], including esters [9], volatile fruity thiols, and monoterpenes [10]. Furthermore, its resistance to osmotic shock is high, making it suitable for high glucose environments, such as ice or botrytized grapes [11]. *Lachancea thermotolerans* (LT) is another important yeast with high lactic acid production [12], low volatile acidity, and low production of unpleasant odor active compounds [13,14]. It is often used to improve the organoleptic properties of wines with low acidity in warm regions to enhance the roundness and balanced acidity [15]. Li Feng et al. found that *H. uvarum* and *L. thermotolerans* were the dominant species in spontaneous fermentations of icewine produced in the Qilian Region of China [16].

So far, there have been several reports on mixed fermentations involving *Saccharomyces cerevisiae* and non-*Saccharomyces* yeasts in wine production, mainly to improve the sensory properties and aroma characteristics [17,18]. However, there is very little information about the production of 'Semillon' icewine using pure culture fermentation or mixture fermentation of indigenous non-*Saccharomyces* yeasts. Therefore, this study aimed to investigate the impact of non-*Saccharomyces* yeasts on the acidity, aroma composition, and sensory quality of 'Semillon' icewine. To that purpose, two non-*Saccharomyces* yeasts stains, *Lachancea thermotolerans* and *Torulaspora delbrueckii*, were chosen and vinified under pure culture and sequential inoculations. The aroma profile and sensory characteristics of the icewines produced using these strains were determined and compared to an icewine fermented with only one *S. cerevisiae* strain.

2. Materials and Methods

2.1. Yeast Strains and Culture Media

The stains of *Lachancea thermotolerans* (LT-2) and *Torulaspora delbrueckii* (TD-3) were isolated from spontaneously fermented must in the Gansu Qilian wine Region (China) by researchers from the Key Laboratory of Viticulture and Enology of Gansu Province and identified by sequencing of the D1/D2 domain of the 26S rRNA genes. Both strains were chosen based on their heterogeneity in phenotypic features, high resistance to wine stress, and good fermentation performance during fermentation. The commercial *Saccharomyces cerevisiae* (SC) strain (Aroma White) was purchased from Enartis, Italy.

The non-*Saccharomyces* yeast strains were routinely grown in YPD medium (10 g/L yeast extract, 20 g/L bacteriological peptone, and 20 g/L dextrose) with or without agar (20 g/L) at 27 °C for 48 h. They were then inoculated in sterilized grape juice medium (50% grape juice and 50% Milli-Q water) with 2% inoculum for subsequent inoculation tests. Wallerstein laboratory nutrient agar medium (WLN) was provided by Auboxing Biotechnology (Beijing, China) and used for viable cell counts. These non-*Saccharomyces* yeast strains were stored at −80 °C in YPD medium with glycerol (25% *v/v* final concentration).

2.2. Chemicals and Standards

Sodium hydroxide, hydrogen chloride, sodium acetate, and acetic acid, all in analytical purity, were purchased from Tianjin Komiou Chemical Reagent Co, Ltd. (Tianjin, China). L-lactic acid and L-malic acid (chromatographically pure) were provided by Shanghai Yuanye Biological Technology (Shanghai, China), while potassium metabisulfite was supplied by Beijing Chemical Works (Beijing, China). The internal standards used for volatile compound quantification were provided by Sigma-Aldrich (Shanghai, China), including ethyl acetate, octanol, isoamyl acetate, 2,3-butanediol, ethyl lactate, ethyl hexanoate, β-damascenone, ethyl octanoate, diethyl succinate, phenethyl acetate, ethyl decanoate, hexanol, phenyl

ethanol, 3-methyl-1-butanol, isobutanol, linalool, *cis*-3-hexen-1-ol, heptanol, butyric acid, decanoic acid, octanoic acid, citronellol, geraniol, nerol, nerolidol, geranyl acetone, 2-octanol, and benzaldehyde.

2.3. Icewine Samples Fermentation Trials

Semillon (*V. vinifera* L.) grape juice was collected in January 2020 from Qilian Winery (Gaotai, Zhangye, Gansu, China). The juice had the following composition: 342.9 g/L of sugar (as reducing sugar), 6.75 g/L of titratable acidity (as tartaric acid), pH of 3.76, 42.0 mg/L of total sulfur dioxide, and 5.1 mg/L of free sulfur dioxide. These oenological parameters were determined based on the methods described by OIV-MA-AS313-01: R2015 [19]. The juice was clarified by adding 100 mg/L $K_2S_2O_5$ and 20 mg/L pectinase at 4 °C for 72 h. The clarified juice was then transferred to 2.5 L brown glass fermenters (2 L per flask). The yeast strains were inoculated as follows: (i) a single inoculation of *L. thermotolerans* (final population of around 7 log CFU/mL after inoculation, (LT)); a single inoculation of *T. delbrueckii* (10^7 CFU/mL, (TD)); and a single inoculation of *S. cerevisiae* (10^7 CFU/mL, (SC)) employed as the control; (ii) a sequential inoculation of *L. thermotolerans*, followed by *S. cerevisiae* after 6 days (10^7 CFU/mL, L-S)); and a sequential inoculation of *L. thermotolerans*, followed by *T. delbrueckii* after 6 days (10^7 CFU/mL, (L-D)). The fermentation temperature was controlled at 14 to 15 °C (corresponding to the winery fermentation temperature), and the fermentations were performed in triplicate. When the alcohol content reached 10 to 11%, the temperature was reduced to less than 2 °C, and 120 mg/L of SO_2 was added to terminate the fermentation. The icewine samples were centrifuged to remove the wine lees and bottled, and part of the samples was stored at −20 °C for analysis.

2.4. Oenological Parameters Analysis

The oenological parameters, including alcohol, volatile acidity, total sugar, and total acidity, were determined based on the methods described by OIV (OIV-MA-AS313-01: R2015, 2015). The glycerin content was determined using a glycerin test kit in a Y15 automatic wine analyzer. High-Performance Liquid Chromatography (HPLC) equipped with a 410 series autosampler, 210 series pump, diode array detector, and a BDS HYPERSILC18 column (250 mm × 4.6 mm, 5 μm) was used for the determination of lactic acid according to the method described by Pérez-Ruiz et al. [20] and Buglass and Lee [21], with slight modifications.

2.5. Fermentation Kinetics and Yeast Biomass

The progress of fermentation was monitored by residual sugar and ethanol measurements. Residual sugar and ethanol contents were measured using a Multi-Function Wine Analyzer (WineScan SO_2 analyzer (FOSS Analytical A/S, Denmark). At a 2-day interval, the residual sugar content was measured until a concentration of 150–160 g/L was attained. The ethanol content of the fermenting must was also determined until 10 to 11% *v/v* was reached, and the fermentation was stopped by adding potassium metabisulfite.

Yeast populations were measured by plate counts after inoculation in Wallerstein Laboratory nutrient (WLN) agar (Sigma-Aldrich), which was mixed with 100 mg/L chloramphenicol (Merck, Darmstadt, Germany). After 48 h of incubation at 28 °C, the colonies were differentially counted based on the morphological color characteristics on the 3rd and 5th days of growth in the WLN culture medium [22,23]. The LT yeast was observed to have a progressive dark green color on the WLN agar, TD yeast with white color, and SC yeast with a creamy yellow color. The biomass was also calculated based on the color of the colony.

2.6. Volatile Aroma Compounds Analysis

Volatile compounds were identified and quantified as described by Gao et al. [24], with slight modifications. Wine aliquots were first subjected to solid-phase microextraction

(SPME) to extract volatile compounds. The wine's volatile compounds were then analyzed by gas chromatography–mass spectrometry (GC–MS). A DB-WAX (60 m × 2.5 mm × 0.25 µm) chromatographic column was used. The column heating temperature started at 40 °C for 7 min, then increased to 200 °C at 4 °C/min and kept for 8 min. Helium was used as the carrier gas and flowed at a rate of 1.2 mL/min. The GC oven temperature started at 40 °C for 5 min, then raised to 200 °C at 4 °C/min, and was maintained for 10 min. The mass spectrometer operated in electron ionization at 70 eV with an ion source temperature of 200 °C and quadrupole temperature of 150 °C, scanning within a mass range from 50 to 350 *m/z*.

2.7. Sensory Analysis

Sensory evaluation was performed 3 months after the end of the fermentations following the procedure described by Ma et al. [25]. The procedure consisted of a sorting task in which the panelists were first asked to smell each wine's odor and then sort the wines into groups based on similar olfactory characteristics. The groups could be formed by as many wines as decided by each panelist, including one single wine, with no limits set for the number of groups. A total of 20 panelists (ten women and ten men) evaluated the wines (6 wine samples). The wine samples (20 mL each) were labeled with random three-digit codes and presented simultaneously to the panelists. The wine samples were served at room temperature in 215 mL ISO standard (ISO) tasting glasses.

2.8. Data Analysis

Excel 2016 (Microsoft, Redmond, WA, USA) and Origin 9.0 (OriginLab, Inc., Northampton, MA, USA) were used for data organization and processing. SPSS 23.0 software (SPSS, Inc., Chicago, IL, USA) was used to perform a one-way analysis of variance on the data. Duncan's multiple comparisons was used to determine significant differences at a confidence interval of 0.05. Additionally, a principal component analysis (PCA) was carried out to determine the association between the detected aroma compounds and the wine samples.

3. Results and Discussion

3.1. Fermentation Process

The icewine samples were fermented at 15 °C, and the fermentation was stopped when the alcohol content reached 10 to 11%. At these alcohol levels, each icewine sample took a different time to complete alcoholic fermentation. The progression of fermentation is shown in Figure 1. Fermentation of the icewine samples by a single inoculation with SC, LT, and TD took 14 d, 22 d, and 26 d, respectively, to reach the alcohol concentration of 10 to 11%. Fermentation of the icewine samples through sequential inoculation with L-S and L-D took 16 d and 22 d, respectively. Thus, the fermentation time of the SC and L-S wine samples was the fastest among all the samples. TD fermented wine was the slowest. The fermentations involving two strains of non-*Saccharomyces* yeasts significantly prolonged the time of alcoholic fermentation. Studies have shown that both *T. delbrueckii* and *L. thermotolerans* have a moderate alcohol fermentation ability [12,26,27]. In this experiment, the strain TD required a longer time to complete alcoholic fermentation. This is beneficial, because a long fermentation time and a slow fermentation rate favor the release of wine aroma compounds [28]. Furthermore, *L. thermotolerans* and *T. delbrueckii* used in single or sequential inoculation resulted in icewines with ethanol concentrations of 10 to 11% *v/v*, which is required for icewine. Nevertheless, both yeast strains required different times to complete alcoholic fermentation.

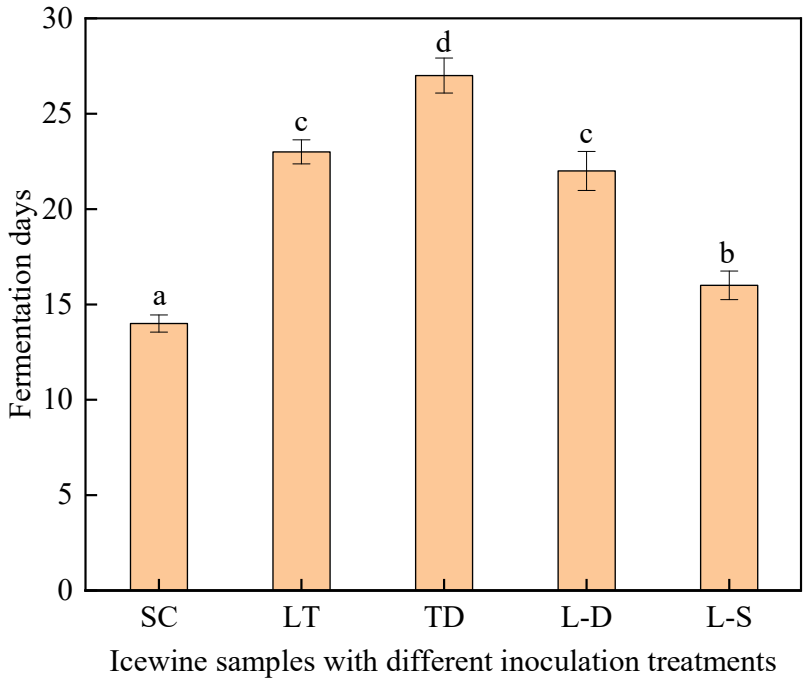

Figure 1. Time used to complete alcoholic fermentation. SC: *S. cerevisiae* pure fermentation; TD: *T. delbrueckii* pure fermentation; LT: *L. thermotolerans* pure fermentation; L-D: sequential fermentation of *L. thermotolerans* and *T. delbrueckii*; L-S: sequential fermentation of *L. thermotolerans* and *S. cerevisiae*. Vertical bars represent standard deviation. Bars with different letters (a, b, c, and d) are significantly ($p < 0.05$) different.

3.2. Yeast Population Dynamics and the Sugar Consumption Trend

The dynamics of yeast growth during alcoholic fermentation are shown in Figure 2A–E. Due to the high initial sugar content of the iced grape juice and the low fermentation temperature, fermentation would stagnate if the amount of yeast inoculated was low. Therefore, the initial cell concentration of ice must after inoculation was 10^7 CFU/mL. This high population level is considered adequate to contribute to the sensory profile of wine [29]. In the early stages (days 4–6) of fermentation, the highest number of yeast cells (7.00–7.58 log CFU/mL) was observed in fermentations with a single inoculation with *S. cerevisiae* (SC) (Figure 2A), while the highest number of cells (7.65 log CFU/mL) was recorded on days 6–8 in LT fermentations (Figure 2B). However, after that, the number of cells decreased slightly at both SC and LT single inoculations. The TD strain's population growth trend was similar to that of LT. The cell population of TD reached the highest (7.56 log CFU/mL) on day 6 and decreased to a final population of 5.5 log CFU/mL. The persistence of yeasts associated with the yeast population during fermentation affects the development of the final aroma characteristics of the wine [30].

During fermentation with sequential inoculation, the population of LT increased rapidly during the first 6 days after inoculation, reaching a peak on day 6. The population of *L. thermotolerans* decreased slightly after inoculation with *T. delbrueckii* but did not disappear completely. At the end of fermentation, the population of both yeasts remained at 6.0–6.5 log CFU/mL. Lage et al. [31] attributed the loss of non-*Saccharomyces* activity in mixed fermentation to changes in the concentrations of major metabolites (especially ethanol) and reduction in the redox potential due to alcoholic fermentation under anaerobic conditions. In addition, *T. delbrueckii* had a good colonization ability, and its population increased rapidly during the initial phase of fermentation, maintaining a certain amount of biomass throughout the fermentation. Some studies have also reported a strong colonization ability of *T. delbrueckii* during fermentation, as it can inhibit the growth of other microorganisms to some extent, which is desirable to improve the wine quality [32]. A

slight inhibition was also observed in the sequentially inoculated icewine sample L-S. The population of *L. thermotolerans* decreased after inoculation with *S. cerevisiae* on day 2 but did not disappear completely, while *S. cerevisiae* became the dominant yeast. At the end of the fermentation, the number of *S. cerevisiae* was about 10^6 CFU/mL, while the number of *L. thermotolerans* remained about 10^5 CFU/mL. This result differs from previous research findings [17] and could be attributed to the high sugar environment of icewine and the early termination of alcoholic fermentation. However, the explanation for the inhibitory effect needs further investigation.

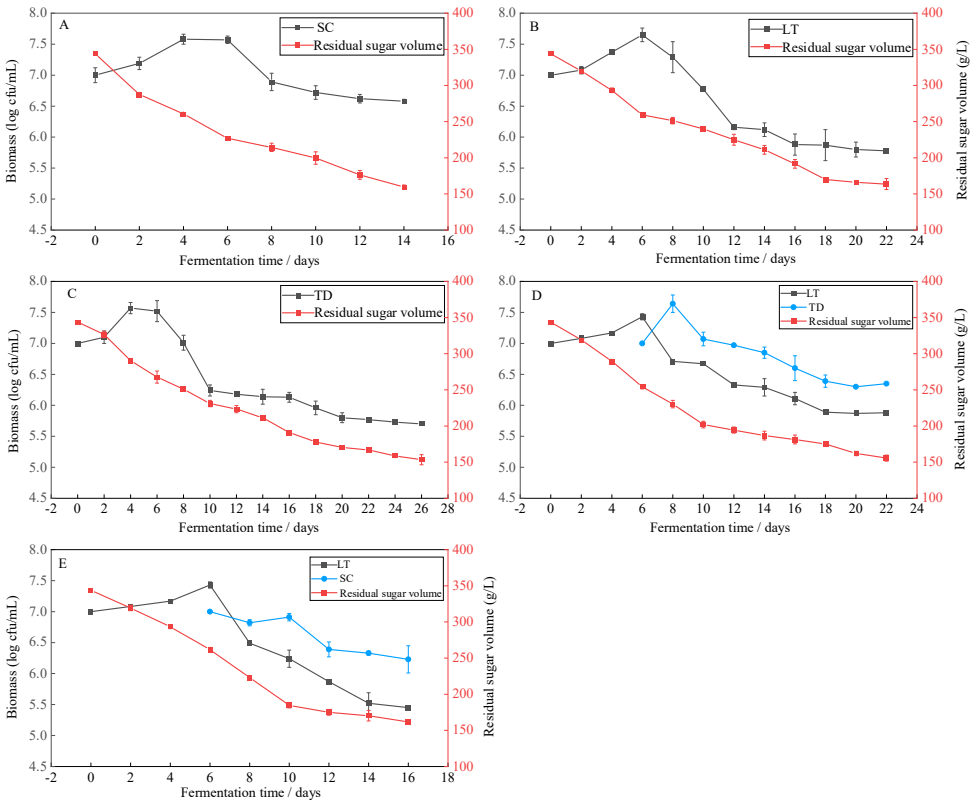

Figure 2. (**A**) Fermentation kinetics and yeast population dynamics fermentations performed by a pure culture of commercial *S. cerevisiae* (SC), (**B**) pure culture of *L. thermotolerans* (LT), (**C**) pure culture of *T. delbrueckii* (TD), (**D**) sequential inoculation of *L. thermotolerans* and *T. delbrueckii* (L-D), and (**E**) sequential inoculation of *L. thermotolerans* and *S. cerevisiae* (L-S).

The initial total sugar content of the must used in this experiment was 342.9 g/L. Single fermentation with *S. cerevisiae* (SC) resulted in a significant decrease in the total sugar content within the first 6 days from 342.9 g/L to 218.0 g/L, while a further reduction to 159.67 g/L was observed on day 14. In contrast, the trend of sugar content decrease was similar in LT and TD fermented icewines. In particular, a rapid decline was observed in LT and TD fermented icewines until the 22nd and 26th days, respectively, when a residual sugar content of 163.67 g/L and 153.33 was reached. Moreover, the decreasing trend of total sugar in L-S icewine was similar to that of LT during the first 6 days of fermentation. After inoculation with *S. cerevisiae*, the sugar content decreased rapidly on day 4 and then decreased steadily until day 16, when the residual sugar content of 161.67 g/L was reached. In contrast, the total sugar content in L-D decreased steadily until day 22, when a residual sugar content of 158.00 g/L was reached. Conclusively, *S. cerevisiae* consumed sugar more rapidly due to its higher ability to ferment sugar and absorb nitrogen than other non-*Saccharomyces* wine yeasts [33–35].

3.3. Oenological Parameters Analysis

The main physicochemical parameters of the 'Semillon' icewines produced by single and sequential fermentations are shown in Table 1. Due to the termination of alcoholic fermentation at 10 to 11% *v/v*, no significant differences were observed in the ethanol concentrations of the icewine samples. However, there were significant differences between the samples regarding residual sugars, ranging from 153.33 to 163.67 g/L. The icewines fermented with the *T. delbrueckii* strain in single inoculations (TD) and sequential inoculations (L-D) had lower residual sugar values (Table 1). This result is consistent with that of Du Plessis et al. [29] and Gobbi et al. [13] and suggests that *T. delbrueckii* consumes more sugar in single and sequential inoculations.

Table 1. Basic physicochemical indexes of icewine samples under different inoculation strategies.

Parameter	Single Fermentation			Sequential Fermentation	
	SC	LT	TD	L-D	L-S
Residual sugar (g/L)	159.67 ± 0.21b	163.67 ± 1.86a	153.33 ± 1.73d	158.00 ± 0.08c	161.67 ± 0.51b
Ethanol (% *v/v*)	10.33 ± 0.15a	10.07 ± 0.15a	10.53 ± 0.45a	10.77 ± 0.23a	10.40 ± 0.53a
Glycerol (g/L)	6.40 ± 0.3d	11.05 ± 0.64c	11.23 ± 1.89c	13.00 ± 0.72a	12.04 ± 0.20b
Acetic acid (g/L)	1.88 ± 0.04a	0.50 ± 0.08d	0.84 ± 0.09b	0.49 ± 0.11d	0.68 ± 0.11c
Total acidity(g/L)	6.57 ± 0.16e	8.08 ± 0.41bc	7.18 ± 0.66d	9.89 ± 0.38a	9.29 ± 0.92b
pH	4.03 ± 0.02a	3.73 ± 0.04c	3.87 ± 0.12b	3.67 ± 0.02c	3.65 ± 0.04c
Lactic acid (g/L)	0.08 ± 0.00d	2.20 ± 0.04a	0.67 ± 0.03c	2.04 ± 0.11b	2.06 ± 0.05b
Malic acid (g/L)	0.46 ± 0.04d	1.15 ± 0.087b	1.41 ± 0.31a	1.09 ± 0.01c	1.15 ± 0.08b

Data are expressed as the means of three samples ± standard deviations. Different letters (a, b, and c) within each column are significantly different (Duncan tests; $p < 0.05$). SC, pure culture fermentation with commercial *S. cerevisiae*; LT, pure culture fermentation with *L. thermotolerans*; TD, pure culture fermentation with *T. delbrueckii*; L-D, sequential inoculation of *L. thermotolerans* and *T. delbrueckii*; and L-S, sequential inoculation of *L. thermotolerans* and commercial *S. cerevisiae*.

Glycerol is an important product of the alcoholic fermentation of yeasts. Higher glycerol levels generally improve the wine quality [36,37]. In this study, the glycerol levels in all icewine samples ranged from 6.40 to 13.00 g/L (Table 1). SC icewine had the lowest glycerol content (6.40 g/L), while the other four icewines had a much higher content (> 11 g/L) than SC. Similar results were reported by Benito et al. [17]. These results also show that *L. thermotolerans* and *T. delbrueckii* can produce a high glycerol content in icewine during fermentation.

Acetic acid is a crucial parameter of icewine, directly contributing to its quality. Due to the high sugar concentration of icewine grape juice, yeast must combat osmotic stress, and yeast cells can use carbon resources derived from sugar metabolism to produce the metabolites necessary for adaptation and survival, such as acetic acid [38]. The amount of acetic acid in all icewine samples ranged from 0.49 g/L to 1.88 g/L. The highest level was found in SC icewine (1.88 g/L), which was below the permissible upper limit for acetic acid (2.1 g/L) allowed in Canadian and Chinese (GB/T 25504-2010) icewines [6]. In contrast, lower levels were found in LT (0.49 g/L) and L-D (0.50 g/L) icewines. The acetic acid content of icewine samples produced with TD was also lower than SC. This result is consistent with the findings of Chen et al. [39], who found that *T. delbrueckii* produces low levels of volatile acidity.

The main objectives of *L. thermotolerans* in winemaking are to increase the acidity and lower the pH by producing L-lactic acid. *L. thermotolerans* strains vary widely in their ability to produce L-lactic acid, ranging from 1.0 g/L to 9.6 g/L, depending on the strain and fermentation conditions [40,41]. Semillon grape juice requires moderate but not too high acidity. Therefore, to moderately increase the acidity of icewine, the use of *L. thermotolerans* strains that produce moderate amounts of lactate is suitable. In our study, the strain LT was selected for its moderate ability to produce lactic acid. As expected, LT icewines had the highest lactic acid content (2.20 g/L), while the purely fermented icewines SC and TD had the lowest (< 1.00 g/L) compared to the other icewines (Table 1). As for malic acid, TD

icewines had the highest content, followed by LT and L-S, while icewines from SC had the lowest content. In addition, icewines produced by single or sequential inoculations with non-*Saccharomyces cerevisiae* yeasts had significantly lower pH than icewines fermented with a single inoculation of *S. cerevisiae*, resulting in a significant increase in the total acidity. Specifically, the total acidity in LT, TD, L-D, and L-S icewines was 1.2 times, 1.1 times, 1.5 times, and 1.4 times higher than in SC. It was observed that acidification (increase in total acidity) by LT resulted in a decrease in pH close of 0.3–0.38 units (Table 1). This value was achieved by producing a maximum of 2–2.2 g/L of lactic acid. Winemakers believe that, at a residual sugar level of 150–160 g/L, the total acidity should be regulated to about 9.0 g/L, which is suitable for a balanced taste of sugar and acidity. Thus, if the residual sugar content of icewine is controlled to 150–160 g/L, fermentation with LT, L-D, and L-S could be a suitable alternative to *S. cerevisiae* in producing icewine with a lower pH and acceptable acidity.

3.4. Analysis of the Main Volatile Compounds in icewines

A total of 70 volatile compounds were detected in the 'Semillon' icewine samples, including 20 esters; 21 higher alcohols; 13 fatty acids; 9 terpenes; and 7 carbonyl compounds (aldehydes, ketones, and other phenylethyl compounds) (Table 2). Thirteen of them had an odor activity value (OAV) greater than one (underlined in Table 2). The threshold values and aroma descriptions of these volatile compounds were taken from the relevant literature [25,42–44]. Compared to the samples from SC, the categories and contents of the volatile aroma compounds differed significantly depending on the type of non-*S. cerevisiae* used and the inoculation method.

Table 2. The concentrations of volatile aroma compounds (μg/L) in 'Semillon' icewines.

No.	Compound	Icewines					OTV (μg/L)	ODE	Reference
		SC	LT	TD	L-D	L-S			
	Esters								
A1	Ethyl acetate	798.96 ± 3.66a	632.4 ± 12.35c	585.24 ± 12.53d	563.34 ± 3.62d	710.41 ± 4.77b	7500	Pineapple, balsam	[25]
A2	Hexyl acetate	72.59 ± 8.99a	1.58 ± 0.84d	5.16 ± 0.56c	12.72 ± 10.76b	13.08 ± 5.48b	1500	Apple, cherries	[42]
A3	Isoamyl acetate	111.44 ± 3.56c	94.23 ± 7.0d	26.37 ± 2.33e	156.90 ± 2.72b	169.54 ± 8.19a	30	Sweet, banana	[25]
A4	Heptyl acetate	3.25 ± 3.25	ND	ND	ND	ND	1400	Rika, Apricot	[25]
A5	Phenethyl acetate	313.65 ± 13.10b	16.38 ± 4.94e	492.66 ± 48.38a	121.96 ± 1.37c	31.26 ± 9.30d	250	Rose, jasmine	[25]
A6	Ethyl butyrate	45.32 ± 37.68a	13.13 ± 3.74c	6.93 ± 4.30d	5.65 ± 4.28e	15.02 ± 7.42b	20	Banana, strawberry	[25]
A7	Ethyl hexanoate	237.83 ± 49.59a	54.14 ± 6.75c	97.46 ± 19.10b	28.76 ± 1.07d	104.91 ± 0.53b	5	Strawberry, apple	[25]
A8	Ethyl heptanoate	12.65 ± 2.35a	ND	1.62 ± 0.43b	ND	ND	220	Pineapple, fruity	[25]
A9	Ethyl octanoate	908.02 ± 9.9a	62.48 ± 4.18e	193.85 ± 30.56c	83.77 ± 3.01d	250.29 ± 8.76b	240	Ripe fruits, pear,	[25]
A10	Ethyl Decanoate	360.3 ± 9.04a	32.74 ± 18.23d	59.86 ± 26.00c	53.03 ± 6.23c	92.44 ± 30.95b	200	Pleasant fruity	[25]
A11	Diethyl succinate	2.84 ± 1.51a	ND	1.22 ± 2.18b	0.76 ± 1.45d	0.95 ± 0.41c	200,000	Fruity, cheese	[25]
A12	Ethyl trans-4-decenoate	16.09 ± 2.97d	23.30 ± 15.75c	196.67 ± 57.50a	24.06 ± 20.34c	89.71 ± 1.23b	/	/	
A13	Ethyl Myristate	9.15 ± 0.94a	2.38 ± 1.34c	3.85 ± 1.52b	4.10 ± 1.31b	1.99 ± 0.45c	500	Mild waxy, soapy	[25]
A14	Ethyl hexadecanoate	3.57 ± 0.63a	1.07 ± 0.06b	0.46 ± 0.92d	0.74 ± 0.17c	0.70 ± 0.02c	1500	Apple, pineapple	[25]
A15	Ethyl propionate	12.79 ± 2.93a	ND	7.02 ± 6.02b	0.52 ± 0.21c	ND	10	Pineapple	[44]
A16	Ethyl dodecanoate	82.64 ± 0.24a	ND	ND	32.51 ± 14.4b	1.76 ± 0.71c	1500	Honey, sweety, fruity	[44]
A17	Ethyl lactate	ND	8.25 ± 0.02b	ND	13.45 ± 0.15a	8.23 ± 2.21b	1500	Fruity	[42]
A18	3-Methylbutyl decanoate	6.21 ± 0.54a	1.71 ± 0.26b	ND	0.98 ± 0.21d	1.28 ± 0.04c	/	/	[44]
A19	Methyl salicylate	2.39 ± 1.08b	1.85 ± 0.72c	3.11 ± 0.67a	1.82 ± 0.15c	ND	40	/	[44]
A20	2-Phenylethyl propanoate	ND	1.87 ± 0.72b	88.55 ± 10.78a	ND	ND	220	Pineapple, fruity	
	Total	2999.69	947.51	1770.03	1105.07	1491.57			
	Higher alcohols								
B1	Propanol	239.9 ± 5.88a	107.98 ± 3.56d	103.44 ± 49.83d	134.02 ± 5.17b	120.10 ± 6.05c	50,000	Fruity	[43]
B2	Isobutanol	278.2 ± 2.79a	140.14 ± 7.69b	111.51 ± 14.95d	120.18 ± 3.09c	65.91 ± 8.54e	75,000	Fusel oil	[42]
B3	1-Butanol	16.50 ± 5.18d	47.82 ± 15.26c	21.93 ± 10.27b	20.64 ± 13.35b	15.29 ± 5.07c	150,000	Medicinal, resinous	[42]
B4	1-Penten-3-ol	4.91 ± 0.57a	2.60 ± 0.41b	2.62 ± 1.72b	2.23 ± 1.69c	2.18 ± 1.44c	/	/	
B5	1-Pentanol	3209.74 ± 0.78b	3773.13 ± 1.30a	3392.15 ± 2.78b	9.32 ± 1.09c	3886.97 ± 5.54a	8000	Balsamic, bitter almond	[25]
B6	Isoamyl alcohol	2091.88 ± 8.83a	ND	ND	ND	666.80 ± 41.20b	30,000	Bitter almond	[43]

Table 2. *Cont.*

No.	Compound	Icewines					OTV (μg/L)	ODE	Reference
		SC	LT	TD	L-D	L-S			
B7	4-Methyl-1-pentanol	ND	3.52 ± 0.15a	ND	ND	2.39 ± 0.84b	50,000	Almond, toasted	[42]
B8	Heptanol	37.73 ± 6.85a	11.76 ± 0.19c	13.96 ± 0.92b	8.14 ± 0.86d	6.77 ± 1.40e	200	Grass, oily	[25]
B9	2-Nonanol	2.24 ± 0.67a	1.68 ± 0.03c	ND	1.59 ± 0.21c	1.83 ± 0.67b	58	Unpleasant floral	[43]
B10	2,3-Butanediol	43.85 ± 20.5a	8.89 ± 2.02c	8.04 ± 1.93c	15.56 ± 9.81b	6.67 ± 4.39c	120,000	Aromatic plant	[42]
B11	1-Octanol	3.61 ± 0.56c	3.50 ± 0.81c	5.33 ± 0.35a	3.88 ± 1.85b	3.54 ± 0.35c	900	Jasmine, lemon	[25]
B12	2-Heptanol	ND	0.43 ± 0.29a	ND	0.48 ± 0.12a	ND	200	Rubbery	[44]
B13	3-Methyl-1-pentanol	5.40 ± 1.18b	10.30 ± 3.71a	4.74 ± 2.86c	5.30 ± 3.22b	4.93 ± 1.71c	50,000	Herbaceous, cocoa	[42]
B14	3-Ethoxypropanol	10.65 ± 1.65b	2.70 ± 1.38e	16.71 ± 1.45a	7.05 ± 1.43c	3.98 ± 2.05d	/	Green, citrus	
B15	Cis-2-Hexen-1-ol	1.08 ± 0.38b	0.98 ± 0.01b	ND	0.29 ± 0.34c	3.80 ± 0.22a	/	/	
B16	4-Penten-1-ol	2.71 ± 0.88b	2.14 ± 0.04c	1.92 ± 1.32c	6.11 ± 4.33a	ND	/	Slight fruity aroma	
B17	1-Decanol	8.06 ± 2.89a	ND	ND	ND	0.59 ± 0.54b	400	Waxy, fatty	[25]
B18	1-Hexanol	339.29 ± 53.56c	514.16 ± 20.8a	486.84 ± 76.71b	363.40 ± 8.51c	305.94 ± 21.50c	8000	Herbaceous, woody	[25]
B19	3-Hexen-1-ol	15.51 ± 3.56a	11.75 ± 3.16b	10.05 ± 4.05c	ND	6.58 ± 1.08d	400	Orange, fruity	[25]
B20	Benzyl alcohol	6.00 ± 0.94a	4.53 ± 0.39c	4.88 ± 2.36b	4.72 ± 2.22b	5.08 ± 3.37b	620	Almond, walnut	[44]
B21	Phenethyl alcohol	1031.40 ± 1.84d	1304.41 ± 2.20c	1912.83 ± 1.85a	1602.6 ± 1.61b	1177.65 ± 1.08c	86	Floral, rose, honey	[25]
	Total	7347.04	5952.42	6096.95	2305.51	6287			
	Acids								
C1	Acetic acid	681.43 ± 7.87a	42.07 ± 1.67c	65.20 ± 2.90b	24.41 ± 10.72d	59.81 ± 11.17b	200,000	Acetic	[42]
C2	Isobutyric acid	13.28 ± 1.26a	2.19 ± 0.33e	6.01 ± 1.02b	5.37 ± 1.76c	4.29 ± 1.67d	8100	Acetic	[42]
C3	Butyric acid	9.40 ± 1.73a	3.79 ± 2.24c	ND	5.68 ± 1.67b	4.05 ± 1.22c	173	Sour, cheese, fatty	[44]
C4	2-Methylbutyric acid	35.64 ± 3.10a	3.20 ± 0.42e	5.21 ± 0.40d	8.04 ± 4.10c	10.77 ± 6.05b	50	Fatty, rancid, cheesy	[44]
C5	Decanoic acid	134.08 ± 7.77a	9.41 ± 1.39d	69.03 ± 0.23c	68.63 ± 1.67c	82.61 ± 2.24b	1400	Butter, cheese	[25]
C6	Hexanoic acid	322.52 ± 1.81a	44.06 ± 4.27d	77.41 ± 12.72c	44.36 ± 4.12d	110.57 ± 4.15b	420	Cheese, fatty	[25]
C7	9-decenoic acid	231.50 ± 7.4a	ND	33.44 ± 4.38c	67.35 ± 7.02b	63.29 ± 6.00b	/	Acetic acid smell	
C8	Octanoic acid	659.75 ± 8.49a	21.03 ± 1.03d	83.24 ± 38.38c	33.08 ± 6.61d	297.78 ± 6.59b	500	Rancid, cheese, fatty	[25]
C9	3-Hydroxylauric acid	1.43 ± 0.46a	0.54 ± 0.02c	0.76 ± 0.98b	0.50 ± 0.48c	0.58 ± 0.41c	/	Sour smell, fatty	
C10	Heptanoic acid	2.21 ± 0.23a	ND	ND	ND	1.48 ± 0.22b	/	/	
C11	17-octadecynoic acid	ND	1.22 ± 0.91a	1.05 ± 0.72b	0.82 ± 0.11c	0.32 ± 0.06d	/	/	

Table 2. *Cont.*

No.	Compound	Icewines					OTV (μg/L)	ODE	Reference
		SC	LT	TD	L-D	L-S			
C12	Trans-3-decanoic acid	ND	4.53 ± 2.02a	ND	ND	ND	/	/	
C13	Pentanoic acid	ND	20.84 ± 0.72a	ND	ND	ND	/	/	
	Total	2091.24	152.88	341.35	258.24	635.46			
	Terpenes								
D1	Linalool	12.11 ± 2.7c	15.57 ± 0.40b	12.27 ± 1.95c	18.72 ± 0.03a	15.58 ± 2.16b	615	Floral, musk	[25]
D2	α-Terpineol	ND	3.50 ± 0.58d	5.98 ± 0.09c	7.67 ± 2.87a	6.74 ± 1.99b	250	Floral	[25]
D3	Citronellol	ND	24.15 ± 3.66d	28.56 ± 6.29c	56.53 ± 9.45a	38.17 ± 8.76b	30	Lemon, citrus, rose	[25]
D4	Nerol	ND	1.71 ± 0.60b	1.02 ± 2.36c	2.47 ± 2.36a	2.69 ± 1.34a	400	Rose, lemon	[42]
D5	Geraniol	9.29 ± 1.98c	14.22 ± 0.64b	5.40 ± 0.33d	9.53 ± 5.40c	15.51 ± 6.98a	30	Peach, floral, musk	[42]
D6	Nerolidol	1.94 ± 1.94bc	1.58 ± 0.93d	2.93 ± 1.95b	5.71 ± 1.95a	2.09 ± 0.60c	700	Apple, rose	[42]
D7	cis-3-Hexen-1-ol	15.11 ± 6.56c	12.79 ± 1.10d	17.96 ± 7.11b	18.87 ± 5.26a	8.48 ± 0.02e	400	Plant, fruity, aromatic	[25]
D8	β-damascenone	2.03 ± 0.12c	1.45 ± 0.3d	2.67 ± 0.03b	2.88 ± 5.90b	11.15 ± 0.82a	0.5	Rose, floral	[25]
D9	Myrcene	1.18 ± 0.45d	1.94 ± 1.23b	0.57 ± 0.09e	1.38 ± 0.47c	2.52 ± 0.45a	/	/	
	Total	41.66	76.91	77.36	123.76	102.93			
	Others								
E1	Benzaldehyde	1.29 ± 0.21c	3.27 ± 0.26a	1.28 ± 0.20c	ND	2.12 ± 0.14b	0.35	Bitter almond	[43]
E2	Decanal	3.94 ± 0.35a	2.27 ± 2.27b	ND	2.28 ± 0.59b	ND	1000	Fruity, floral	[43]
E3	Methylheptenone	4.05 ± 14.14a	0.91 ± 0.27c	ND	1.22 ± 3.99b	0.86 ± 0.19c	/	Rose, plum	
E4	Damastone	17.56 ± 4.43e	22.38 ± 2.58c	20.73 ± 2.37d	34.27 ± 7.99b	37.59 ± 3.22a	0.05	/	[44]
E5	3-Hydroxy-2-butanone	ND	4.17 ± 1.41a	ND	2.75 ± 1.59b	1.37 ± 0.62c	150,000	Peach, plum	[43]
E6	Geranyl acetone	ND	ND	1.03 ± 0.73b	2.11 ± 0.89a	1.10 ± 0.53b	60	Floral	[44]
E7	Styrene	6.06 ± 0.54a	1.79 ± 0.05d	2.02 ± 0.77c	2.91 ± 0.36b	ND	/	Pungent	
	Total	32.9	34.79	25.06	45.54	43.04			

Data are the means ± standard deviation. Different letters (a, b, c, d, and e) within each row are significantly different (Duncan tests; $p < 0.05$). "ND" indicates not detected. "/" means not found. The references [25,42–44] represents relevant literature from which the threshold values and aroma descriptions of the volatile compounds were taken from. Twenty odor active compounds (OVA > 0.1) were underlined. ODE, odor descriptor; OTV, odor threshold value; SC, pure culture fermentation with commercial *S. cerevisiae*; LT, pure culture fermentation with *L. thermotolerans*; TD, pure culture fermentation with *T. delbrueckii*; L-D, sequential inoculation of *L. thermotolerans* and *T. delbrueckii*; and L-S, sequential inoculation of *L. thermotolerans* and commercial *S. cerevisiae*.

3.4.1. Esters

Esters are important wine components, because they can impart fruity and floral aromas to wines, increase the aroma complexity of wines, and contribute to the uniqueness of the wine body [45]. Depending on the yeast strains used in fermentation, the concentrations and types of esters can vary widely [46]. The total concentration of esters (947.51 μg/L) in LT fermented icewines was the lowest of all treatments, which may be related to the metabolism of *L. thermotolerans*. The ester concentrations in icewines produced by sequential fermentation ranged from 1105.07 to 1491.57 μg/L, which was significantly lower by 50.28%–63.16% compared with SC icewines (2999.69 μg/L). In all icewine samples, rose-flavored phenethyl acetate was significantly higher in TD than in other icewines [25].

As for the sequential fermentations, L-S had higher ester concentrations than L-D. Ethyl lactate, which is related to lactic acid metabolism and may positively contribute to wine sensory characteristics by improving the aroma complexity [39], was not found in the SC and TD samples. In addition, ethyl dodecanoate, known for its earthy, smoky, spicy, dried fruit, and roasted aroma [47], was found in L-S and SC fermented icewines, with SC fermented wines having a significantly higher amount (82.64 μg/L) compared to L-S icewines (1.76 μg/L).

3.4.2. Higher alcohols

Higher alcohols are formed as byproducts of alcoholic yeast fermentation. They are synthesized mainly by the Ehrlich pathway, in which amino acids are used as substrates for transamination and decarboxylation before being reduced by alcohol dehydrogenase. In addition, yeasts can also form higher alcohols from carbon fragments of sugar metabolism [30]. Studies have shown that higher alcohols of less than 300 mg/L can lead to pleasant aromas in wine [39], while higher concentrations (400 mg/L) can have a negative impact on the aroma [48]. The total concentration of higher alcohols in all icewine samples ranged from 2305.51 to 7347.04 μg/L and contributed positively to the wine aroma profile. The highest total concentration of higher alcohols was found in sample SC, followed by samples L-S, TD, LT, and L-D. The total higher alcohols in icewines fermented with non-*S. cerevisiae* ranged from 14.43% to 68.62%. Fermentation with *L. thermotolerans* was reported to reduce the production of higher alcohols compared to fermentation with *S. cerevisiae* [48]. Similarly, a significant decrease in the total concentration of higher alcohols was observed in white wines produced from sequential fermentations of Emir grape must with *L. thermotolerans* and *S. cerevisiae* [33]. Phenylethyl alcohol was reported to have the aroma characteristics of honey and roses [49]. It is worth noting that the phenylethyl alcohol content in TD wine samples was significantly higher than in other fermentations, suggesting that using *T. delbrueckii* in fermentation may enhance the aromatic notes of icewine such as honey and rose.

3.4.3. Acids

Acids are byproducts of yeast metabolism and are mainly produced during alcoholic and malolactic fermentations. The commonly perceived cheese and fat taste in wine are closely related to the acids in wine [50,51]. At low concentrations, acids can increase the complexity of wine aromas, while a high concentration can cause astringency and irritation [52]. In this experiment, the total acid content in all icewine samples ranged from 152.88 μg/L to 2091.24 μg/L, with the SC sample having the highest concentration and LT sample having the lowest. In addition, acetic acid, isobutyric acid, butyric acid, decanoic acid, and 9-decanoic acid were detected in varying concentrations, with none exceeding the aroma threshold. The fatty acid concentration of wine samples fermented with TD was significantly lower than SC in this study (Table 2). This was congruent with the findings of Azzolini et al. [9], who discovered a similar effect on fatty acid synthesis, with a two- to four-fold reduction. Additionally, their concentrations were significantly lower in icewines produced by non-*S. cerevisiae* yeasts compared to SC. Despite having lower concentrations compared to SC, these compounds, especially decanoic acid and

octanoic acid, could contribute positively to improving the aroma and sensory quality of icewines, as they are associated with buttery and cheesy odors.

3.4.4. Terpenes

Terpenes can be formed during wine fermentation by yeast metabolism, where glucosidases readily bind to glycosylated precursors, generating terpenic compounds with floral and fruity notes that may contribute positively to the wine aroma [53]. A total of nine terpene aroma compounds were detected in this experiment, including geraniol, β-damascenone, linalool, α-terpineol, citronellol, nerol, and myrcene. These terpenes were significantly higher in LT, TD, L-T, and L-S icewines than in the SC wine samples. It has been reported that geraniol, β-damascenone, and linalool are the major terpenes in icewine [45]. The total concentration of terpenes in wines produced by sequential inoculations L-D and L-S were 123.76 μg/L and 102.93 μg/L, respectively, while that of single inoculations (SC, LT, and TD) ranged from 41.66 μg/L to 77.36 μg/L. The total concentration of terpenes in the sequential inoculation samples (L-D and L-S) was significantly higher than in SC icewine (Table 2). Among all icewine samples, the L-D sample had the highest amount of total terpenes (123.76 μg/L), which was about three times higher than the SC samples (41.66 μg/L). Geraniol, which has a pleasant floral and musky aroma, is an important terpene in wines with a low odor threshold (30 μg/L) and could directly stimulate human olfactory cells. In this experiment, the concentration of geraniol in icewines produced by single and sequential inoculations (LT and L-S) was significantly higher than SC. Nevertheless, the concentrations in all samples exceeded the odor threshold, which could contribute floral and fruity aromas to the icewines. In one study, geraniol production by *L. thermotolerans* was higher than that of other yeast species [53]. Citronellol, α-terpineol, and nerol have also been reported to contribute floral and fruity notes to wines [53]. Interestingly, these terpenes were not detected in SC icewines. β-damascenone in the L-S sample was similarly higher than in SC. C_{13}-norisoprenoid compounds are derived from the degradation of carotenoids in grapes, with β-damascenone being the most important in non-aromatic grape varietals [54]. β-damascenone was the only C_{13}-norisoprenoid compound detected in this study. Its concentration significantly increased in L-S wines (Table 2), which was enough to contribute its floral notes [52] to the overall aroma because of its high OAV.

3.4.5. Others

The total content of aldehydes, ketones, and other compounds in all icewine samples differed significantly ($p < 0.05$) and ranged from 25.06 μg/L to 45.54 μg/L. Benzaldehyde, which has the taste of bitter almonds, was found in all wines except L-D. Moreover, 3-hydroxy-2-butanone was found only in LT, L-S, and L-D but at low concentrations. In addition, very low concentrations of geranyl acetone and methylheptenone were detected in L-S icewine. Although the levels of these compounds were extremely low, they may contribute floral and plum aromas to the icewine.

3.5. Principal Component Analysis of Volatile Aroma Compounds

PCA was performed on aroma compounds with OVA > 0.1. The first two principal components, PC1 and PC2, accounted for 22.0% and 56.5% of the total variance, respectively. The loading of the aroma categories and the distribution of the wine samples among the first two principal components are shown in Figure 3. Icewines fermented with non-*S. cerevisiae*, such as L-S and LT, were on the positive side of PC2, while L-D and TD were on the negative side of PC2. In addition, 1-pentanol, ethyl hexanoate, ethyl decanoate, octanoic acid, heptanol, ethyl octanoate, 2-methylbutyric acid, ethyl propionate, and hexanoic acid were on the positive side of PC1 and mainly contributed to the aroma profile of SC icewine. Moreover, these compounds distinguished the wine fermented with a pure culture of *S. cerevisiae* (SC) from the pure and sequentially fermented icewines without *S. cerevisiae* (LT, TD, L-S, and L-D), which were mainly associated with geraniol, β-damascenone, citronellol, benzaldehyde, phenethyl alcohol, 2-phenethyl propanoate, and damastone on the negative

side of PC1 (Figure 3). These compounds, particularly geraniol, β-damascenone, citronellol, damastone, and benzaldehyde, had odor activity values greater than 0.1 and may have imparted to LT, TD, L-S, and L-D icewines their floral and fruity notes [53].

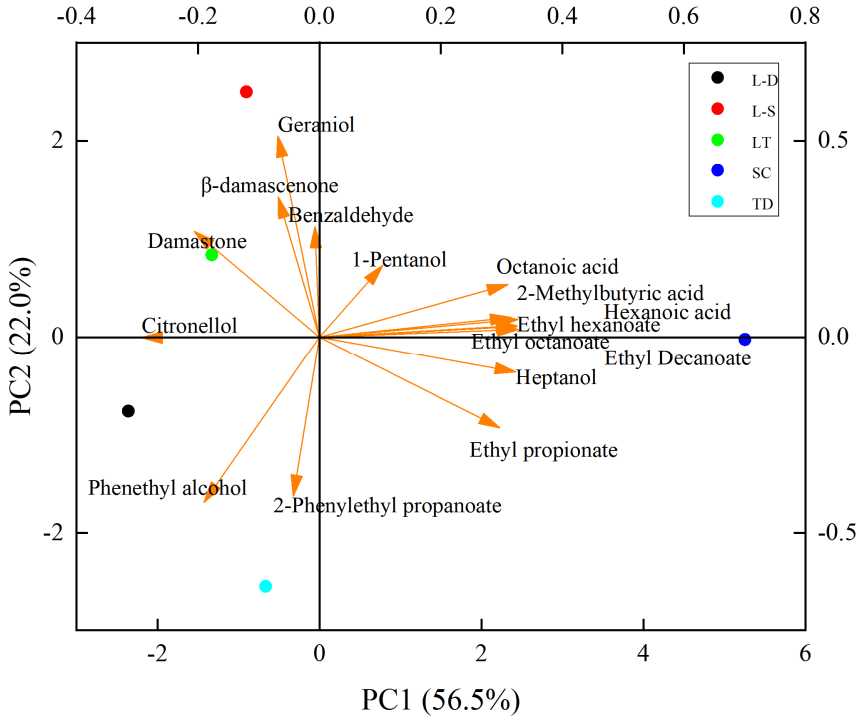

Figure 3. Principal component analysis of volatile compounds (OAV > 0.1) in icewine samples with different inoculation treatments. SC, pure culture fermentation with commercial *S. cerevisiae*; LT, pure culture fermentation with *L. thermotolerans*; TD, pure culture fermentation with *T. delbrueckii*; L-D, sequential inoculation of *L. thermotolerans* and *T. delbrueckii*; and L-S, sequential inoculation of *L. thermotolerans* and commercial *S. cerevisiae*.

3.6. Sensory Evaluation

In this experiment, the sensory analysis showed that some sensory characteristics differed significantly between the icewine samples (Figure 4). Non-*Saccharomyces* yeasts can improve the aroma complexity of icewines by producing more terpenes and phenethyl compounds during fermentation [55], which was evident in our sensory results. It was found that L-S icewine had the highest score for all sensory attributes (except fruity notes), while L-D icewine had a more intense fruity aroma than other samples. Regarding the aroma intensity, L-S wine had the highest score. In addition, there were no differences between samples L-D and TD, but significant differences were found when compared to samples SC and LT. This is related to the esters and aromatic alcohols with fruity aromas, such as ethyl lactate and phenethyl ethanol, released during sequential inoculation, as well as to the higher concentrations of terpenes, aldehydes, and ketones that confer a rich floral and fruity aroma and uniqueness to the wines (L-S, L-D, and TD). Morata et al. [55] and Benito et al. [17] also obtained similar results, in which *L. thermotolerans* and *S. cerevisiae* were inoculated sequentially. Furthermore, all icewine samples had similar colors, clarity, and peculiarity. Nevertheless, the perception of acidity was high in the L-S and L-D wines, which had higher values than the other treated wines. Thus, no difference was found in the balance of these two icewines.

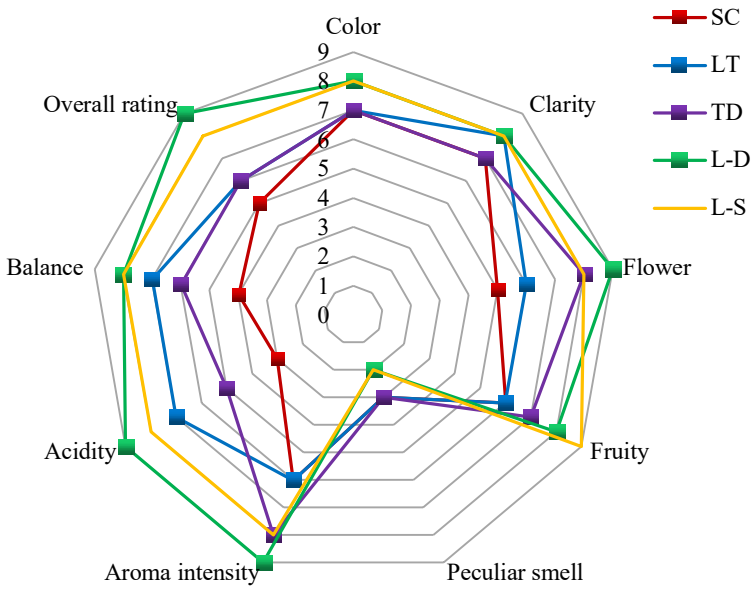

Figure 4. Radar map of the sensory analysis of icewines produced with different fermentation strategies. SC, pure culture fermentation with commercial *S. cerevisiae*; LT, pure culture fermentation with *L. thermotolerans*; TD, pure culture fermentation with *T. delbrueckii*; L-D, sequential inoculation of *L. thermotolerans* and *T. delbrueckii*; and L-S, sequential inoculation of *L. thermotolerans* and commercial *S. cerevisiae*.

4. Conclusions

In this study, the effects of different inoculation strategies, such as single inoculation and sequential inoculation with *S. cerevisiae*, *L. thermotolerans*, and *T. delbrueckii*, were investigated in the production of 'Semillon' icewine. In sequential inoculation, the growth of *L. thermotolerans* was slightly inhibited by the presence of the *S. cerevisiae* or *T. delbrueckii* strains. Single or sequential inoculations with *L. thermotolerans* and *T. delbrueckii* resulted in a significant decrease in volatile acidity, an increase in the glycerol content, and a significant decrease in the concentration of esters and terpenes. In addition, the fermentations with *L. thermotolerans* yielded relevant amounts of lactic acid. The sensory analysis also showed that the sequentially fermented icewines L-S and L-D exhibited more fruity notes, floral odor, and aroma intensity. Therefore, *L. thermotolerans* and *T. delbrueckii* could be used in sequential fermentations to improve the aroma quality of icewines.

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