

Article

# Assessing the Oenological Potential of *Nakazawaea ishiwadae*, *Candida railenensis* and *Debaryomyces hansenii* Strains in Mixed-Culture Grape Must Fermentation with *Saccharomyces cerevisiae*

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**Abstract:** Recently, there has been a growing interest in the role of non-*Saccharomyces* yeast (NSY) as a coculturing partner with *Saccharomyces cerevisiae* during grape must fermentation. We investigated three new strains, namely *Nakazawaea ishiwadae*, *Candida railenensis* and *Debaryomyces hansenii*, for their oenological potential in mixed-culture micro-vinifications with *S. cerevisiae* Vin13 using Muscaris grape must. None of the NSY strains impeded the fermentation performance as all the mixed-culture experiments finished at the same time. Coculturing with *N. ishiwadae* yielded significantly higher concentrations of ethyl and acetate esters in the final wine product. Apart from higher acetic acid levels, wines produced with *C. railenensis* and *D. hansenii* yielded much lower esters concentrations. The concentrations of certain terpenes and norisoprenoids were also significantly modulated in the mixed-culture fermentations. This study reveals the rarely reported species of *N. ishiwadae* as a promising coculturing partner for increasing aroma-active compounds in a wine.

**Keywords:** non-*Saccharomyces* yeast; *Nakazawaea ishiwadae*; aroma; mixed-culture fermentations; wine yeast

## 1. Introduction

Since the second half of the 1960s, commercial starter cultures of *Saccharomyces cerevisiae* wine yeast strains became available and soon afterwards were widely adopted among the winemaking community. Using starter cultures allowed for a far more reliable fermentation with far fewer reports of stuck or sluggish fermentations [1]. Different kinds of wine yeast were also developed for specific outcomes and varieties of grapes; yet, they all belonged to the *Saccharomyces* genus. A large-scale genomic analysis of different wine yeast revealed that many commercial yeast are, from a genomic perspective, remarkably similar [2], and it was suggested that breeding with *S. cerevisiae* strains outside of the “wine clade” would be necessary in order to expand on the winemaking capabilities of wine yeast, including to develop on the breath of possible flavor profiles for wines prepared using defined cultures.

Alternatively, wine yeast researchers have, for the past twenty years, started to investigate the use of non-*Saccharomyces* yeasts (NSY; also called non-conventional yeasts) as starter culture co-partners with *S. cerevisiae*. Several of these investigations resulted in the successful coculturing of *Saccharomyces* and non-*Saccharomyces* and yielded wines with improved qualities. Examples of improved characteristics included a marked decrease in ethanol levels, increased release of varietal

aroma compounds, favorable acid modulation, increased aroma complexity and higher polysaccharide presence as a result of the coculturing of an NSY with *S. cerevisiae* [3–6].

Some of the coculturing experiments led to the availability of several commercial NSY starter cultures. These include strains of *Lachancea thermotolerans*, *Metschnikowia pulcherrima*, *Pichia kluyveri*, *Schizosaccharomyces pombe*, *Starmerella bacillaris* and *Torulaspota delbrueckii*. All of these NSY were isolated from oenological environments and through various trials have been shown to provide consistent improvements to various aspects of wine. However, there are many other NSY yeasts isolated from wine that are yet to be fully explored in terms of their compatibility with *Saccharomyces* wine yeasts and their potential contribution to wine quality [5,7,8]. In the context of ongoing assessments of the oenological potential of NSY yeasts, some progress have been made with experiments using species of *Issatchenkia* [9,10] and *Hanseniaspora* [11,12]. Coculturing studies with selected strains of both of these genera indicated that they have the capacity to contribute positively to the final wine; however, these NSY yeasts are yet to make it to the market.

In this study, we explored the utility of three NSY yeasts with limited knowledge about their co-fermenting contributions—*Nakazawaea ishiwadae*, *Candida railenensis* and *Debaryomyces hansenii*—in mixed-culture Muscaris grape must fermentations.

## 2. Materials and Methods

### 2.1. Grape Must

Muscaris grapes from the 2018 vintage harvested in a vineyard of the Hochschule Geisenheim University in the Rheingau wine region of Germany were used for micro-vinifications. The grape juice's starting sugar content was 215.4 g/L (105.3 g/L glucose and 110.1 g/L fructose) with the two major wine acids, namely tartaric acid and malic acid, being 4.7 g/L and 3.8 g/L, respectively. The must's primary amino acid content was assayed using the NOPA (nitrogen by *o*-phthaldialdehyde) method [13] and was determined to be 121 mg/L. The free ammonium level was determined to be 69 mg/L using a Rapid Ammonium kit from Megazyme (Bray, Ireland).

### 2.2. Selection of Strains

The yeasts of unknown identity isolated from vineyards belonging to Geisenheim University were initially isolated on YGCB agar plates (5 g/L yeast extract, 20 g/L glucose, 0.2 g/L chloramphenicol, 0.01 g/L bromophenol blue, 2 g/L agar). Sixty of these strains were plated on solid medium (2 g/L) containing 50% Muscaris grape must added to the agar after autoclaving and allowed to grow for two days at 25 °C. The agar plates were subjected to a "sniffing test" where eleven members of the Institute of Microbiology and Biochemistry at Geisenheim University surveyed the plates by conducting a simple olfactory inspection. Genomic DNA was isolated from six strains that exhibited an overall agreeable aroma to the panel. The genomic DNA was used to conduct an identification PCR test by amplifying the 5.8S-ITS (internal transcribed spacer) region using the ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) primer pair. PCR products were purified using a sodium acetate/isopropanol method, and their respective DNA sequences were determined with StarSeq (Mainz, Germany) and were aligned using the program Muscle, which is included in MEGA version 5 [14].

### 2.3. Micro-Vinification

The three NSY yeasts were cultivated overnight in 100 mL YPD (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) at 30 °C. The cells were harvested and then resuspended in 100 mL autoclaved Muscaris must and cultivated overnight. Approximately  $1 \times 10^7$  cells/mL (determined with a hemocytometer) were used to inoculate 220 mL of Muscaris must (only pasteurized) in a 250 mL wine bottle capped with an airlock and incubated at 22 °C. The same preculture procedure was followed with *S. cerevisiae* Vin13 (a widely used commercial wine yeast from Anchor Yeast, Cape Town), which was aseptically added to wine bottles after three days at a concentration of  $1 \times 10^6$  cells/mL. Fermentations were deemed

finished once no weight-loss was recorded over a 24-hr period. Cells were removed via centrifugation and wine samples were stored at 4 °C prior to analysis.

#### 2.4. HPLC-Analysis

To determine the initial concentration of the main sugars and acids of the grape must as well as the final concentrations of the major metabolites in the wine, high-performance liquid chromatography (HPLC) was employed using a modified method described previously [15]. An Agilent Series 1100 HPLC, equipped with a binary pump, an autosampler, a multi-wavelength detector (MWD) and a refractive index (RI) detector (Agilent Technologies, Steinheim, Germany) was used. An Allure Organic Acids column (Restek, Bad Homburg v. d. Höhe, Germany) with a particle size of 5 µm, an inside diameter of 4.6 mm and a column length of 250 mm was used. The MWD was set at a wavelength of 210 nm for the detection of organic acids and the RI was used for the detection of carbohydrates, organic acids and ethanol. The eluent used was water with 0.5% ethanol and 0.139% concentrated sulfuric acid. The flow rate was 0.6 mL/min at a temperature of 46 °C (sugars, ethanol and organic acids) and 29 °C (glycerol). The data was processed using Chemstation software (Agilent, Steinheim, Germany).

#### 2.5. Volatile Compound Analysis

Wine samples of 5 mL each were subjected to headspace solid phase microextraction gas chromatography mass spectrometry (HS-SPME-GC-MS) analysis to assess the quantities of various aroma compounds. A GC 6890N was equipped with an MS 5977B (both Agilent, Santa Clara, CA, USA) and an MPS II robotic autosampler and CIS 4 (both Gerstel, Mülheim an der Ruhr, Germany). Ten microliters of an internal standard (1-octanol) was added to the wine sample and 1.7 g of NaCl was placed into a 20-mL headspace vial. Solid-phase microextraction was carried out with a 65-µm polydimethylsiloxane and divinylbenzol fiber (Supelco, Merck, Darmstadt, Germany). Aroma compound separation was performed using a 60 m × 0.25 mm × 1 µm gas chromatography column (Rxi<sup>®</sup>-5Si1, Restek, Bad Homburg v. d. Höhe, Germany) with helium used as a carrier gas. The sample was injected in split mode (1:10, initial temperature 30 °C, rate 12 °C/s to 240 °C, hold for 4 min). The GC run started with an initial temperature of 40 °C for 5 min, then raised to 210 °C at 5 °C/min, and then raised again to 240 °C at 20°/min and held for 10.5 min. Mass spectral data were acquired in a range of mass to charge ratio ( $m/z$ ) of 35 to 250 and used to derive concentration values. Calibrations were performed with a model wine 3%, 6%, 9% or 12% ( $v/v$ ) solution of ethanol in water with the pH adjusted to 3 g/L tartaric acid and the pH adjusted to 3.0). Terpene and norisoprenoid analysis were conducted as detailed previously [16].

#### 2.6. Statistical Analysis

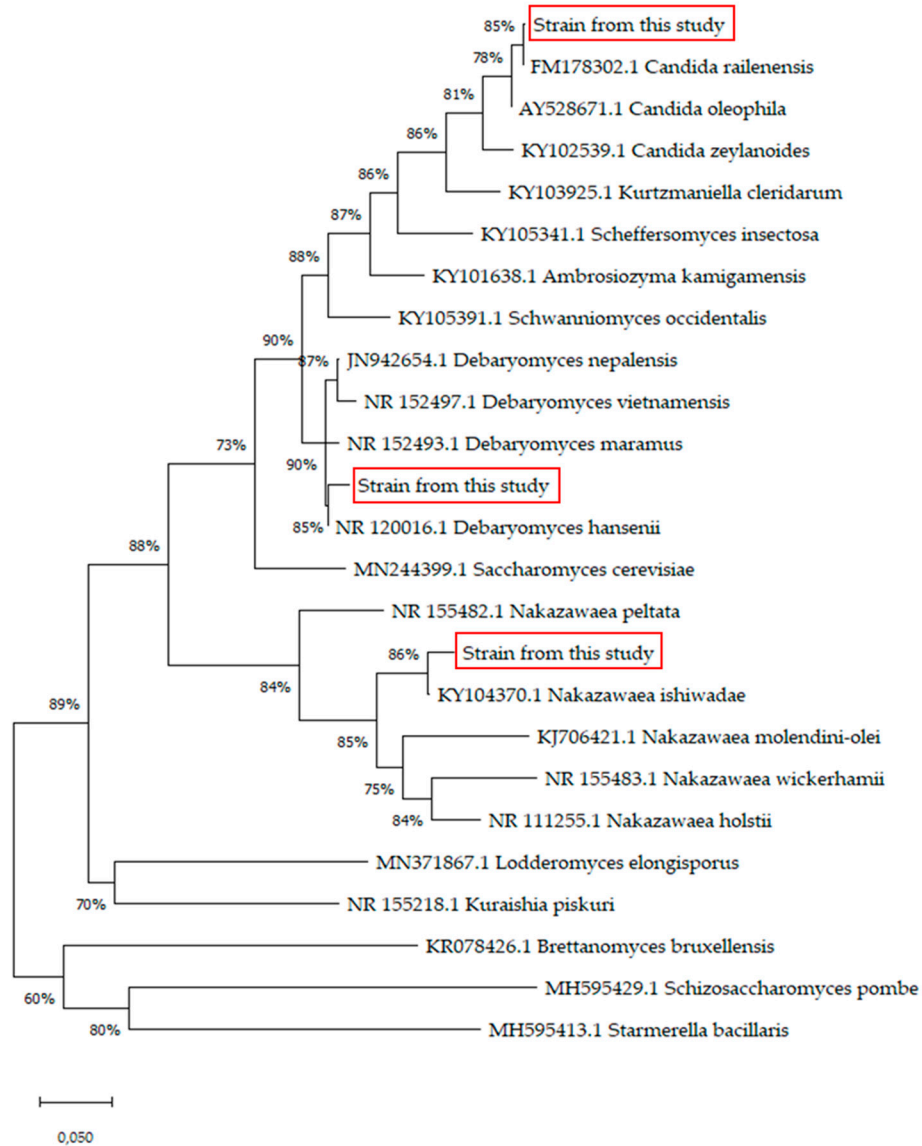
The fermentations were conducted in biological triplicates. All comparisons of the data generated from HPLC and GC-MS were made against the control (fermentations inoculate with Vin13 only) using One-way ANOVA with Dunnett's post-hoc test using GraphPad Prism version 5.00 for Windows, (GraphPad Software, San Diego, CA, USA).

### 3. Results and Discussion

#### 3.1. Strain Identification

Six of the sixty strains tested via olfaction inspection were identified by the panel as imparting an overall pleasant aroma. Based on their 5.8S-ITS region two of the six strains that were identified as *Debaryomyces hansenii*, two as *Metschnikowia pulcherrima*, one as *Candida railenensis* and one as *Nakazawaea ishiwadae* (Figure 1). *D. hansenii* and *M. pulcherrima* strains are commonly isolated from wine milieus [5]. Since many coculturing attempts have been made with *M. pulcherrima* showing its utility in a coculturing must fermentation [17–19], it was decided to exclude it from further coculturing

experiments. Despite a common member of the wine yeast population, *D. hansenii* strains have not been explored to a large extent in mixed-culture fermentations. Only one of the *D. hansenii* isolates were used for coculturing experiments as their ITS sequence were identical and it was assumed they would perform similarly.

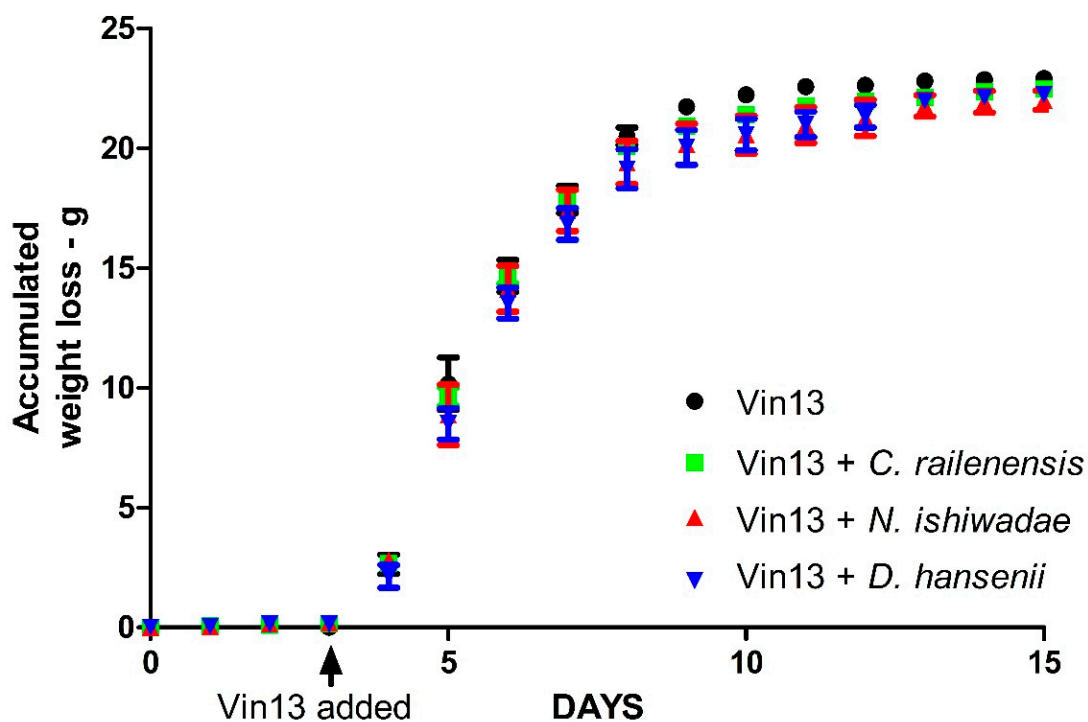


**Figure 1.** Maximum likelihood analysis showing the placement of the non-*Saccharomyces* species used in the study among common wine and closely related yeasts, including using their ITS1–5.8S–ITS2 region. The dendrogram was based on the general time reversible model (GTR + G + I) as determined with MEGA 5.2 [14,20]. Bootstrap percentages from a 1000 bootstrap replicates are shown. Bar, 5% sequence divergence.

Although far less known, *C. railenensis* and *N. ishiwadae* have also been isolated from vineyards and wineries in previous studies [21–24]. *N. ishiwadae* has been tested in a mixed-culture fermentation in a synthetic must, showing an increased release of proteins into the wine [22]. *C. railenensis* was an initial candidate tested for increased thiol release from Sauvignon Blanc grape must [23], but, to the best of our knowledge, this is the first account of this yeast being used in a mixed-culture fermentation experiment.

### 3.2. Fermentation

A sequential fermentation procedure was followed where the must was fermented for three days with the respective NSY culture before adding the *S. cerevisiae* Vin13 strain. No meaningful weight-loss was observed prior to the addition of Vin13. The NSY cultures also had little influence on the fermentation performance as there was no significant difference in the weight-loss profiles of the mixed-culture fermentations when compared to the control ferment inoculated with Vin13 only (Figure 2). Little to no difference in many of the oenological parameters in the mixed-culture fermentations was observed compared to the Vin13 control (Table 1). There was, however, a slight increase of approximately 20% in acetic acid levels with the mixed-culture fermentations with *C. railenensis* and *D. hansenii*. With *N. ishiwadae*, a 12% drop in glycerol levels was observed, although it did not correspond with an increase in ethanol levels as there was no significant modulation in ethanol levels. Although a minor acid in wine, there was a noteworthy reduction in the shikimic acid levels in the *N. ishiwadae* mixed-culture fermentation.



**Figure 2.** Fermentation kinetics measured as the daily accumulation of weight loss of the three mixed-culture fermentations conducted in triplicate. Error bars indicate the standard deviation. Once no weight loss was recorded over a period of one day, fermentations were considered finished.

### 3.3. Volatile Composition

There are two main ways by which yeast can impart flavor and aroma to a wine fermentation [25]. The first mechanism involves the production of aroma-active compounds that form part of a yeast's primary and secondary metabolism. The second mechanism is when a yeast strain produces enzymes like glycosidases or carbon-sulfur lyases that are capable of releasing aroma-active compounds from bound precursors present in grape must, which contribute toward the so-called varietal aroma. Using different of NSYs, previous coculturing experiments have demonstrated that NSY can impact both ways. This is especially the case with imparting increased varietal aromas, as many *S. cerevisiae* strains lack the enzymes needed for the release of volatile compounds from their bound moieties.



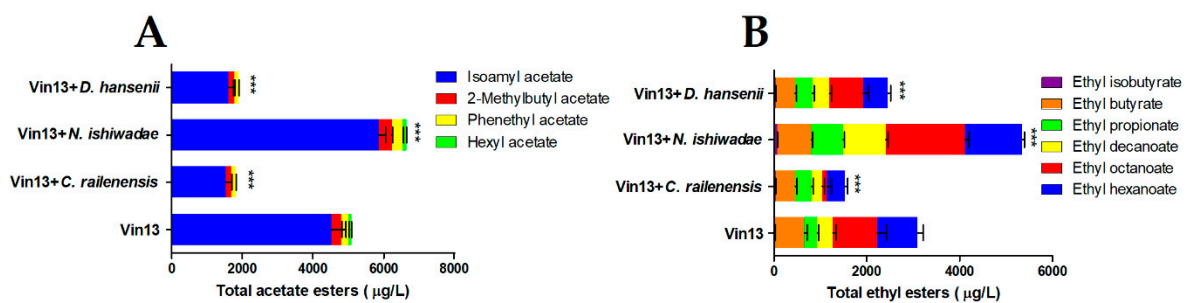
**Table 1.** Analytical results of the main oenological parameters resulting from the mixed-culture fermentations.

	Residual Sugars	Tartaric Acid	Malic Acid	Acetic Acid	Citric Acid	Lactic Acid	Shikimic Acid	Glycerol	Ethanol
Vin13 (alone)	nd	3.7 ± 0.0	3.1 ± 0.0	0.42 ± 0.0	0.22 ± 0.0	0.18 ± 0.0	0.008 ± 0.0	6.97 ± 0.1	12.57 ± 0.2
Vin13 (+ <i>C. railenensis</i> )	nd	<b>4.0 ± 0.1</b> <b>0.1 ***</b>	3.1 ± 0.0ns	<b>0.51 ± 0.02</b> <b>0.0 **</b>	0.21 ± 0.0ns	0.17 ± 0.0ns	0.008 ± 0.0ns	6.90 ± 0.1ns	12.73 ± 0.3ns
Vin13 (+ <i>N. ishiwadae</i> )	2.4 ± 0.1	<b>4.0 ± 0.1</b> <b>0.1 **</b>	3.0 ± 0.1ns	0.45 ± 0.0ns	0.22 ± 0.0ns	0.17 ± 0.0ns	<b>0.002 ± 0.001</b> <b>0.0 ***</b>	<b>6.20 ± 0.1</b> <b>0.1 ***</b>	12.70 ± 0.2ns
Vin13 (+ <i>D. hansenii</i> )	2.1 ± 0.0	<b>4.0 ± 0.1</b> <b>0.1 ***</b>	<b>3.0 ± 0.1</b> <b>0.1 *</b>	<b>0.52 ± 0.02</b> <b>0.0 **</b>	0.21 ± 0.0ns	0.17 ± 0.0ns	0.008 ± 0.0ns	<b>6.67 ± 0.1</b> <b>0.2 *</b>	12.53 ± 0.2ns

All values in g/L and are the average from a triplicate with the “±” sign indicating the standard deviation. (nd not detected; ns not significant; \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ). Boldface indicate significant values.

Upon assessing the volatile content in the wines derived from the mixed-culture fermentations, significant differences in the ester content (both acetate and ethyl ester) were observed compared to the Vin13 control fermentation, as shown in Figure 3 and Table 2. With the *N. ishiwadae* coculturing, all the esters showed a marked increase compared to the Vin13 control (a 24% increase in acetate esters minus ethyl acetate and a 42% increase in ethyl esters). A previous *N. ishiwadae* mixed-culture fermentation also showed an increase in the ester content [24]. The strain, on its own, did not finish the fermentation. For both *C. railenensis* (a 64% decrease in acetate esters and 49% decrease in ethyl esters) and *D. hansenii* (a 62% decrease in acetate esters and 21% decrease in ethyl esters) mixed-culture fermentations, a dramatic reduction in ester content was observed. Of note is that no hexyl acetate was detected in both *C. railenensis* and *D. hansenii* cocultures. The lower ester levels could be ascribed to enhanced esterase activity by these two strains that presumably hydrolyzed esters back to their respective higher alcohols. In a previous study, strong esterase activity was reported for a *D. hansenii* strain [26]. The hydrolysis of the acetate esters by esterases could also have contributed to the increased acetic acid found in wines derived from the *C. railenensis* and *D. hansenii* mixed-culture fermentations.

Muscaris is a fungus-resistant grape variety related to the aromatic Muscat variety with a high content of terpenes [27]. In Table 3, we show the concentrations of the terpenes and norisoprenoids in the final wines. The mixed-culture fermentations with *C. railenensis* and *D. hansenii* showed significantly higher levels of both versions of rose oxide, whereas *C. railenensis* coculturing also resulted in a 3.3-fold increase in citronellol levels. *D. hansenii* is known for its  $\beta$ -glucosidase activity, which is capable of releasing terpene compounds bound to sugar moieties, as has been previously demonstrated [28]. Little is known about the glycanases of *C. railenensis*. Interestingly, inexplicably lower levels of the two most common terpenes in Muscaris, namely hotrienol and  $\alpha$ -terpineol, were measured in the *D. hansenii* fermentation.



**Figure 3.** Sums of the (A) acetate esters (excluding ethyl acetate) and (B) ethyl esters in the final wines derived from the mixed-culture fermentations. Fermentations were done in triplicate. Error bar represents the standard deviation for each ester; \*\*\*  $p < 0.001$ .

**Table 2.** Aroma volatiles in the final wines derived from the mixed fermentations as determined with GC-MS. Values are the average from a triplicate with the “±” sign indicating the standard deviation. The compounds 2-hydroxy-4-methylvaleric acid ethylester, succinic acid diethylester, benzeneacetic acid ethylester and 2-methylbutyric acid methylester were measured during the analysis, but were not detected in any of the samples. ns not significant \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ . Boldface indicate significant values.

	Aroma Descriptor	Vin13 (alone)	Vin13 (+ <i>C. railenensis</i> )	Vin13 (+ <i>N. ishiwadae</i> )	Vin13 (+ <i>D. hansenii</i> )
$\beta$ -myrcene	Pepper, spicy	6.47 ± 1.8	6.09 ± 1.9ns	6.58 ± 2.1ns	3.98 ± 3.5ns
limonene	Citrus, orange	1.10 ± 0.1	1.02 ± 0.2ns	1.06 ± 0.1ns	1.12 ± 0.1ns
<i>cis</i> -rose oxide	Rose	1.05 ± 0.0	<b>2.11 ± 0.2 ***</b>	1.24 ± 0.0ns	<b>1.92 ± 0.1 ***</b>
<i>trans</i> -rose oxide	Rose	0.29 ± 0.0	<b>0.56 ± 0.0 ***</b>	0.34 ± 0.0ns	<b>0.51 ± 0.0 ***</b>
<i>cis</i> -linalool oxide	Floral	83.20 ± 4.5	75.70 ± 3.4ns	77.18 ± 2.6ns	80 ± 8.5ns
<i>trans</i> -linalool oxide	Floral	42.29 ± 1.8	37.74 ± 2.2ns	41.86 ± 1.5ns	39.63 ± 4.8ns
nerol oxide	Green, herbal	12.82 ± 0.1	12.93 ± 0.7ns	<b>11.11 ± 0.2 *</b>	12.62 ± 0.8ns
vitispirane	Floral, fruity	0.62 ± 0.0	<b>0.60 ± 0.0 *</b>	0.61 ± 0.0ns	<b>0.66 ± 0.0 **</b>
linalool	Floral, rose	75.00 ± 1.3	<b>85.55 ± 4.8 *</b>	78.63 ± 0.4ns	<b>84.12 ± 4.6 *</b>
hotrienol	Sweet, tropical	148.40 ± 1.1	<b>160.20 ± 9.1 *</b>	140.1 ± 4.2ns	<b>101.4 ± 7.5 **</b>
$\alpha$ -terpineol	Pine, terpene	220.60 ± 5.5	238.60 ± 17.2ns	217.3 ± 9.4ns	<b>153.6 ± 11.5 ***</b>
citronellol	Floral, citrus	2.34 ± 0.6	<b>7.80 ± 0.3 ***</b>	<b>5.49 ± 1.2 **</b>	3.82 ± 0.4ns
$\beta$ -damascenone	Fruity, floral	0.79 ± 0.0	<b>1.00 ± 0.1 **</b>	<b>1.04 ± 0.0 **</b>	0.90 ± 0.1ns

**Table 3.** Terpene and norisoprenoid composition of the Muscaris wines derived from the mixed-culture fermentations as determined with GC-MS.

	Aroma Descriptor	Vin13 (alone)	Vin13 (+ <i>C. railenensis</i> )	Vin13 (+ <i>N. ishiwadae</i> )	Vin13 (+ <i>D. hansenii</i> )
<b>Higher alcohols</b>					
2-Methyl-butanol (mg/L)	Roasted, winery	68.67 ± 10.0	88.67 ± 2.9 *	74.33 ± 11.2ns	86.00 ± 4.4ns
Isobutanol (mg/L)	Ethereal, winery	78.00 ± 16.6	64.67 ± 4.0ns	78.33 ± 18.9ns	65.33 ± 1.2ns
Isoamyl alcohol (mg/L)	Fusel, alcoholic	335.3 ± 49.4	391.7 ± 15.0ns	348.7 ± 55.6ns	369.7 ± 13.6ns
Hexanol (mg/L)	Herbal, ethereal	790.7 ± 74.1	882.0 ± 29.7ns	<b>616.0 ± 56.3 **</b>	868.3 ± 7.4ns
2-Phenyl-ethanol (mg/L)	Floral, rose	26.00 ± 2.0	31.00 ± 1.7ns	26.67 ± 3.1ns	31.00 ± 3.5ns
<b>Acetate esters</b>					
Ethyl acetate (mg/L)	Ethereal, fruity	99.00 ± 9.8	<b>72.67 ± 5.5 *</b>	<b>245.0 ± 14.0 ***</b>	76.33 ± 6.0 *
Isoamyl acetate (µg/L)	Banana, fruity	4520 ± 410	<b>1530 ± 170 ***</b>	<b>5858 ± 192 ***</b>	<b>1606 ± 148 ***</b>
2-Methylbutyl acetate (µg/L)	Overripe, fruity	277.0 ± 24.0	<b>153.0 ± 16.8 ***</b>	<b>376.0 ± 10.6 ***</b>	<b>170.3 ± 11.6 ***</b>
Phenethyl acetate (µg/L)	Floral, rose	214.7 ± 11.7	<b>132.0 ± 4.4 ***</b>	<b>311.0 ± 12.2 ***</b>	<b>124.7 ± 4.7 ***</b>
Hexyl acetate (µg/L)	Green, fruity	71.7 ± 8.7	<b>0.0 ± 0.0 ***</b>	<b>99.6 ± 4.0 ***</b>	<b>0.0 ± 0.0 ***</b>
<b>Medium-chain fatty acids</b>					
Isovaleric acid (µg/L)	Cheesy, dairy	1253 ± 6.4	<b>1318 ± 14.6 ***</b>	<b>1316 ± 15.0 ***</b>	<b>1284 ± 3.2 ***</b>
Hexanoic acid (mg/L)	Sour, fatty	5.0 ± 0.0	4.0 ± 0.0ns	5.0 ± 0.0ns	5.0 ± 0.0ns
Octanoic acid (mg/L)	Fatty, waxy	3.0 ± 0.0	3.0 ± 0.0ns	3.0 ± 0.0ns	2.3 ± 0.6ns
Decanoic acid (µg/L)	Sour, fatty	864.7 ± 7.8	881.0 ± 10.2ns	<b>910.3 ± 6.7 ***</b>	871.7 ± 7.6ns
<b>Ethyl esters</b>					
Ethyl isobutyrate (µg/L)	Ethereal, fruity	30.7 ± 1.5	<b>36.7 ± 1.2 **</b>	<b>74.0 ± 2.6 ***</b>	<b>41.3 ± 1.5 ***</b>
Ethyl butyrate (µg/L)	Sweet, fruity	625.3 ± 60.2	<b>420.7 ± 42.1 ***</b>	<b>737.7 ± 21.2 *</b>	<b>415.0 ± 32.4 ***</b>
Ethyl propionate (µg/L)	Sweet, fruity	281.7 ± 25.3	<b>354.3 ± 34.2 *</b>	<b>690.0 ± 17.0 ***</b>	<b>371.7 ± 37.0 *</b>
Ethyl decanoate (µg/L)	Sweet, waxy	334.0 ± 74.7	<b>226.3 ± 54.1ns</b>	<b>913.7 ± 43.1 ***</b>	370.0 ± 46.1ns
Ethyl octanoate (µg/L)	Fruity, winery	953.3 ± 206.8	<b>104.0 ± 93.0 ***</b>	<b>1710 ± 76.0 ***</b>	727.3 ± 112.1ns
Ethyl hexanoate (µg/L)	Sweet, fruity	870.7 ± 118.6	<b>389.3 ± 60.7 ***</b>	<b>1231 ± 40.3 **</b>	<b>538.7 ± 54.7 ***</b>

Values are the average from a triplicate with the “±” sign indicating the standard deviation.  $\beta$ -ionone, nerol, myrtenol, 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN) and 1,8 cineol were also measured but were either not quantifiable or detectable in any of the samples. All concentrations in µg/L. (ns not significant \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ). Boldface indicates significant values.

#### 4. Conclusions

We have tested the possible oenological gain of three NSYs not often explored in mixed-culture grape must fermentation. Although both *C. railenensis* and *D. hansenii* showed an increased release of

certain terpenes and norisoprenoids, it was the cofermentation with *N. ishiwadae* that showed the most potential, as a notable increase in many ethyl and acetate esters was obtained. Overall, esters impart a pleasant fruity and flowery aroma to a wine and are often desired in young wines. Thus, a higher level of esters would add to the complexity of the wine, although this claim awaits confirmation through sensory analysis. If this observation of coculturing can be repeated with different musts and on a larger scale, this reasonably unknown yeast with no representative whole genome sequence currently available could be included on a list of important NSY starter cultures that are commercially available.

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