YEAST ASSIMILABLE NITROGEN REQUIREMENTS OF INNOCULATED AND SPONTANEOUS FERMENTATIONS OF RIESLING (*V. VINIFERA* L.)

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Camila Martins Tahim

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ABSTRACT

Nitrogen plays a major role in the metabolic processes of fermentative microorganisms, affecting fermentation kinetics and formation of flavor-active compounds. Though a Yeast Assimilable Nitrogen (YAN) concentration of 140 mg N/L is generally recognized as the minimum required to avoid stuck or sluggish fermentations, most research on YAN requirements has focused on warm climate cultivars and conditions, and has been extrapolated for use in other regions. This work aimed to define optimal YAN concentrations for cool-climate fermentations, specifically those of Riesling. Riesling grape juice with YAN concentrations adjusted to 130-300 mg N/L with diammonium phosphate (DAP) additions was fermented with three yeast strains commonly used in the Finger Lakes, NY, wine region. Fermentation kinetics suggested that 130 mg N/L concentration was enough to complete fermentation for the three yeast strains studied, and DAP additions improved fermentation kinetics only for yeast strain EC1118. Analysis of select volatile compounds via GC-MS showed that ester concentrations increased with nitrogen addition, but the unsupplemented control wine was preferred by panelists in a sensory study.

In a separate experiment, spontaneous fermentations of Riesling were monitored in two Finger Lakes wineries to assess their YAN requirements and microbiome composition. Non-*Saccharomyces* yeast species were isolated through late stages of fermentation, and most of the *S. cerevisiae* isolates identified were found to be similar to commercial strains. Fermentation kinetics and microflora were markedly different in the two wineries studied, but in all spontaneous fermentations the YAN consumption range was lower than that in the inoculated fermentations described above.

This work suggests that nitrogen requirements for cool climate Riesling fermentations are moderate (140 mg N/L or lower), and that DAP supplementation should be applied with caution

to avoid excess residual nitrogen and possible negative effects on sensory properties. The initial assessment of spontaneous fermentations indicated that nitrogen requirements are likely lower than inoculated fermentations, precluding the need for nitrogen additions in the winery.

BIOGRAPHICAL SKETCH

Camila Tahim is from Fortaleza, Brazil, and graduated in 2007 with a B.S degree in Food Engineering from University of Campinas – Unicamp. In 2006, she started working as in intern in Procter & Gamble Brazil. There, she worked for 7 years as a Process Engineer for manufacturing lines ranging from cough syrup to baby diapers, and lastly as Supply Chain Manager for the diaper business in Brazil. During that time, she developed a growing appreciation for wine, which is why in 2014 she went on to pursue a Master's degree in Food Science at Cornell University, with a concentration in enology. During her time at Cornell, Camila researched nitrogen requirements of Riesling fermentations and worked in the extension program, under Dr. Anna Katharine Mansfield's supervision.

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CHAPTER 1
YEAST ASSIMILABLE NITROGEN (YAN) OPTIMIZATION FOR COOL-CLIMATE RIESLING

Abstract: Adequate Yeast Assimilable Nitrogen (YAN) concentration is necessary for successful wine fermentation, so supplementing musts with nitrogen is a common industry practice. In the Finger Lakes region of New York, Riesling often has YAN concentrations below the 140 mg/L considered a practical minimal limit. However, little research exists to confirm nitrogen requirements for cool-climate cultivars and conditions. To test the influence of juice nitrogen concentration on desirable sensory characteristics, a Riesling juice of 20° Brix and 130 mg/L initial YAN was supplemented with diammonium phosphate (DAP) to increase YAN to 180, 250 and 300 mg/L. Each supplemented fraction and the unaltered control were inoculated with three different *Saccharomyces cerevisiae* yeast strains: EC1118 (Lallemand), W15 (Lallemand) and Côte des Blancs (Red Star). The control concentration of 130 mg N/L was enough to complete fermentation for the three yeast strains studied, and further supplementation improved fermentation kinetics only for EC1118. Nitrogen supplementation affected the concentration of eight of the 10 select volatile compounds analyzed in at least one of the yeast strains. Of these, the concentration of most esters increased with nitrogen supplementation, with the exception of ethyl cinnamate, which decreased. Concentrations of higher alcohols 1-hexanol and 2-phenylethanol decreased, and decanoic acid increased, with increased nitrogen. Linalool and 1,1,6-Trimethyl-1,2-dihydronaphthalene, two volatiles associated with 'varietal character' in Riesling, were not affected. In a preference ranking test, panelists preferred the unsupplemented control (130 mg N/L) over the supplementation treatments for wines fermented with EC1118. No difference in preference was found for W15 and Côte des Blancs treatments.

Introduction

The macronutrients required by yeast for fermentation are relatively abundant in grape musts. Nitrogen, however, is a key nutrient that is often below optimum concentrations, as shown in worldwide surveys of wine musts (Butzke 1998, Gockowiak and Henschke 1992, Hagen et al. 2008). A variety of nitrogenous compounds are found in grapes, but only some can be assimilated by yeast. Yeast assimilable nitrogen (YAN) is the term for this usable nitrogen fraction, which is defined as the sum of the ammonia (AMM) and primary amino nitrogen (PAN) concentrations (Monteiro and Bisson 1991, Henschke and Jiranek 1993). YAN measurement excludes proteins and peptides, which cannot be metabolized by yeast, and secondary amino acids, most notably proline.

The importance of YAN to fermentation metabolism is well known in the winemaking industry, as YAN concentration and composition is highly variable among grape musts, and can potentially impact the quality of the resulting wine. Early research on nitrogen requirements of *Saccharomyces cerevisiae* established 140 mg N/L as the minimum YAN concentration for the successful completion of most wine fermentations (Butzke 1998; Bell and Henschke 2005). Several authors later suggested that optimal concentrations range from 200 to 350 mg N/L, depending on factors such as initial sugar concentration, yeast strain, and wine style (Bisson and Butzke 2000, Torrea et al. 2011, Miller et al. 2007, Ugliano et al. 2011).

Since YAN deficiencies are known to cause stuck or sluggish fermentations, winemakers routinely supplement musts with diammonium phosphate (DAP). Such additions can significantly affect the production of yeast-derived volatiles, including higher alcohols, esters, fatty acids, sulfur compounds, and organic acids (Bell and Henschke 2005). The pathways by which nitrogen affects the synthesis of volatiles are complex and not completely elucidated. Higher alcohols, for instance, are proposed to be formed via the Ehrlich pathway, which involves

transamination of amino acids, followed by decarboxylation and reduction steps (Torrea et al. 2011). Furthermore, esters can be formed by condensation of the corresponding alcohol and a coenzyme-A activated acid, catalyzed by an acyltransferase (Torrea et al. 2011). Generally, nitrogen additions in nitrogen-deficient must will increase the production of ethyl and acetate esters, and decrease production of higher alcohols (Hernandez-Orte et al. 2006, Miller et al. 2007). Because of these effects, several authors have suggested the use of nitrogen supplementation as a tool to optimize expression of wine aroma (Vilanova et al. 2012, Torrea et al. 2011, Miller et al. 2007, Ugliano et al. 2011).

In addition to the volatiles mentioned above, monoterpenes are key aroma compounds in aromatic cultivars like Riesling and Gewürztraminer. Present in grapes mainly in the form of non-volatile glycoconjugates, monoterpenes can be modified to various degrees during fermentation. Yeast and bacteria produce glycosidase enzymes, liberating the free form of monoterpenes such as linalool, geraniol, nerol, and citronellol (Strauss et al. 1986, Swiegers et al. 2005). Further, it has been demonstrated that some yeast strains can synthetize monoterpenes, and that high YAN concentrations favor this synthesis (Carrau et al. 2005, Vilanova et al. 2012). Yeasts also are likely to play a role in the conversion of precursors into C_{13} norisoprenoids, such as β-damascenone and α- and β-ionone, although this mechanism is not yet well understood (Bell & Henschke, 2005). In Riesling, 1,1,6-Trimethyl-1,2-dihydronaphthalene (TDN), another C_{13} norisoprenoid, reportedly contributes to the typical "petrol" aroma of those wines (Simpson and Miller 1983, Sacks et al. 2012). As with other C_{13} norisoprenoids, TDN is formed from glycosylated precursors by a combination of acid- and enzyme-catalyzed reactions (Winterhalter et al. 1990). Nitrogen status of the must has been shown to affect the formation of β-ionone and

β-damascenone, likely by changing glycosidase activity (Vilanova et al. 2012). Consequently, it can be hypothesized that TDN concentration could also be impacted by YAN concentration.

Most YAN studies have used synthetic media or juices and musts from warm climate regions, making YAN recommendations for cool climate winemaking largely conjectural. Further, because YAN analysis requires specialized equipment, DAP additions are frequently made prophylactically, without knowledge of the original YAN concentration or the final YAN target. This often leads to excessive supplementation, which can have negative consequences on a wine's organoleptic properties, and may result in high levels of residual nitrogen (Bell & Henschke, 2005). In addition to increasing the possibility of microbial instability, excess nitrogen may result in the formation of the known carcinogen ethyl carbamate, and of biogenic amines, which can cause deleterious health effects in susceptible individuals (Daudt et al. 1992, Monteiro et al. 1989).

Among the factors influencing nitrogen requirements of wine fermentations, the strain of *S. cerevisiae* used is one of the most important. The three *S. cerevisiae* strains most widely used for Riesling fermentation in the Finger Lakes region- EC1118 (Lallemand), also known as Prise de Mousse, W15 (Lallemand), and Côte des Blancs or Epernay II (Red Star)- have varying nitrogen needs. In synthetic media fermentations, EC1118 was found to require 140 mg N/L to ferment to dryness, increasing fermentation rates and nitrogen consumption at higher YAN concentrations (Gutierrez et al. 2012). W15 strain is reportedly very similar, if not identical, to AWRI 796 (Maury) (Deed et al. 2011), which required 300 mg N/L to ferment to dryness in a study performed in synthetic media (Vilanova et al. 2007). This is consistent with the manufacturer's classification of W15 as a strain with high nitrogen requirements (Lallemand). The specific YAN requirements of Côte des Blancs are not defined in literature, but it is

described as a slow fermenter, typically displaying a long lag phase and low fermentation rates (Bisson and Butzke 2000).

Riesling is the most widely planted *Vitis vinifera* cultivar of New York State, covering 1,034 acres in 2011, according to the USDA (www.nass.usda.gov/ny). A three-year study conducted in New York vineyards showed that YAN concentrations in this cultivar are generally low, averaging below 100 mg/L each year (Nisbet et al. 2014). Although several studies have characterized the volatile composition of Riesling wines (Bowen and Reynolds, 2012, Komes et al. 2006, Simpson and Miller 1983), the effect of must YAN on wine composition has not been investigated. This study focused on determining the optimum YAN concentration for Riesling fermentations of the Finger Lakes, given the importance of this variety for the region. The effect of nitrogen levels on fermentation kinetics and on the formation of volatile and non-volatile metabolic products was evaluated.

Materials and Methods

Model wine trials. To provide an initial assessment of differences between the yeast strains studied, synthetic wine fermentations were performed using modified chemically defined grape juice (CDGJ) medium (Henschke and Jiranek 1993). The sugar concentration was modified to 200 g/L (100 g/L glucose and 100 g/L of fructose) to better represent average sugar concentrations found in Finger Lakes Riesling (Nisbet et al. 2014). Concentration of amino acids was proportionally reduced to yield an initial PAN concentration of 40 mg N/L. The YAN content was then increased to 80, 140, 220 and 300 mg N/L by adding diammonium phosphate (DAP). Each of those YAN levels was inoculated with four yeast strains: EC1118 (*Lallemand*), Côte des Blancs (*Red Star*), W15 (*Lallemand*) and AWRI 796 (*Mauri Yeast Australia*). Fermentations were performed in duplicate, and samples were taken daily for sugar and YAN

analysis. Once fermentations were complete, the synthetic wines were screened for pH, TA, organic acids and ethanol.

Grape source. Riesling (*V. vinifera* L.*)* grapes were hand harvested at 20°Brix on 24 Oct 2014 at the Cornell University research vineyards in Lansing, NY. Fruit was stored in plastic picking lugs at 3°C, then transported to the Vinification & Brewing Laboratory on 27 Oct 2014, where it was immediately crushed and basket-pressed (Mori press type PZ.82, Impianti Enoligici, Italy) at 200 psi. After pressing, a sulfur dioxide (SO_2) addition of 50mg/L was made using potassium metabisulfite ($K_2S_2O_5$). The juice was settled overnight at ambient conditions, then racked and distributed into 11.4 L carboys for fermentation.

Nitrogen Supplementation. Once the juice was fractioned into the individual fermentation vessels, it was supplemented with DAP to create treatments with 180, 250 and 300 mg N/L of total YAN. All fermentations were performed in duplicate.

Yeast selection. Each YAN treatment and the control juice was fermented with three *S. cerevisiae* yeast strains: EC1118 (*Lallemand*), Côte des Blancs (*Red Star*) and W15 (*Lallemand*). These strains were selected because they are popular for Riesling production in the Finger Lakes, and have different manufacturer-identified nitrogen demands.

Fermentations and Sampling. Fermentations were carried out in 11.4 L carboys, each receiving 10.5kg of juice. Yeast was rehydrated as per manufacturer's protocol; in short, GoFerm (Scott Laboratories, Petaluma, CA) was dissolved in 40°C deionized water and added at 0.3g/kg of juice, contributing 10 mg N/L to the juice YAN. Subsequently, yeast was added at a rate of 0.3 g/kg of juice. After inoculation, each carboy was topped with a three-piece airlock with floating bubbler (Buon 129 Vino Manufacturing Cambridge, ON). Fermentations took place in a controlled temperature room held at 18°C. Every 24h, duplicate 2mL aliquots were taken from

each fermenter using 5mL disposable sterile pipettes (Celltreat, Shirley, MA). The samples were centrifuged at 12,000 rpm for 5 min, and the supernatant removed and stored at -15°C until analysis. After all fermenters had stopped producing CO_2, Clinitest tablets (Bayer Inc, Elkhart, IN) were used to estimate the reducing sugar remaining in the wine. At completion, wines were racked from the lees into 7.5 L (2 gal) carboys, and 60 mg/L of SO_2 was added. The wines were then moved to a 2°C room for cold stabilization. Once the wines were cold stable, they were racked off the tartrate crystals and manually bottled and screw capped in 750mL bottles (Saint-Gobain Packaging, Fairfield, CA).

Samples (2mL) for residual sugar and YAN were taken at the end of fermentation. After cold stabilization, samples were taken for pH, TA and organic acids, and at bottling samples were collected for ethanol analysis.

Analytical methods. The concentrations of glucose, fructose, tartaric acid, malic acid, citric acid and acetic acid were measured by high performance liquid chromatography (HPLC), as described previously (Castellari et al. 2000), on an Agilent 1100 series HPLC (Agilent Technologies, Santa Clara, CA, USA). YAN was determined through the separate enzymatic analysis of ammonia (AMM) and primary amino nitrogen (PAN), using a Chemwell 2910 Multianalyzer (Unitech Scientific, Hawaiian Gardens, CA) to rapidly test samples. AMM concentration was quantified using the glutamate dehydrogenase enzymatic test (Ough 1969). Reagents for this test were supplied in the form of enzymatic kits (Unitech Scientific, Ammonia Extended Range UniTAB, 2007). PAN was determined by the o-phthaldehyde/N-acetyl-L-cysteine spectrophotometric assay (NOPA) procedure (Dukes & Butzke 1998) (Unitech Scientific, Primary Amino Nitrogen UniTAB, 2007).

Wine volatiles were analyzed by gas chromatography-mass spectrometry (GC-MS). TDN, linalool, and several higher alcohols and esters were isolated from the wine using a solid-phase extraction (SPE) protocol referenced in previous studies (Lopez et al. 2002; Sacks et al. 2012). Twenty-five μL of the internal standard (2-octanol, 0.5 g/L in acetonitrile) was added to 50 mL of sample. Samples were loaded onto SPE cartridges (Merck, Darmstadt, Germany) containing 200 mg of LiChrolut EN sorbent preconditioned with 4 mL of dichloromethane, 4 mL of methanol, and 4 mL of model wine (consisting of 12% v/v ethanol and 5 g/L tartaric acid, adjusted to pH 3.5 using NaOH) Cartridges were placed in a 12-piece manifold (J.T. Baker, Center Valley, PA) to facilitate elution, were allowed to dry under N_2 for 20 min, and then analytes were eluted with 1.3 mL of dichloromethane.

GC-MS analyses were conducted on an Agilent 6890 gas chromatograph with a split−splitless injector (Santa Clara, CA) coupled to an Agilent HP 5973 Mass Selective Detector. Separation was performed using an Agilent DB-5MS column (30m x 250mm i.d. x 0.25μm). The initial oven temperature was 35 °C and held for 3 min, then ramped to 200 °C at 6 °C/min, then to 240 °C at 30 °C/min, and held at 240 °C for 3 min. The GC was operated at a constant flow rate of 1 mL/min. One microliter of extract was injected splitless. The injector temperature was 250 °C and had a purge time of 1.00 min (Purge flow 50ml/ min; Inlet pressure 68.9kPa). The auxiliary channel, set point quadruple, and set point source were 280, 150, and 230, respectively. Helium was used as the carrier gas. Data processing and quantification were performed using Agilent Enhanced MSD ChemStation software (G170EA E.02.00.493).

Identification of linalool was confirmed by comparing the retention time and mass spectra to the authentic standard. For the nine additional compounds analyzed, identification was made through comparison of the Kovats retention index (KI) and mass spectra with those of

commercial standards. After retention times and mass spectra were determined, detection took place using selected ion scan mode (SIS) to increase sensitivity (Table 1.9).

A full quantification was made for linalool by preparing a calibration curve (n=4), in duplicate, in model wine with a concentration range of 8-220 μg/L and r^2= 0.9096. In the absence of a pure TDN standard, the calibration curve was made with naphthalene (n=4), with a range of 1-170 μg/L, and r^2= 0.9922. The TDN concentration was then determined in naphthalene equivalents. Naphthalene is commonly used as an internal standard for TDN determinations via GC-MS, due to the similarity in the chemical structures of the two compounds (Daniel et al. 2009).

A semi-quantitative approach was taken for esters, higher alcohols and organic acids. For each treatment, relative response factors (RRF's), defined as the ratio between the peak areas of the analyte and internal standard, were compared to the RRF's of the respective control.

Sensory evaluation. Wines were evaluated for preference by a sensory panel consisting of 31 participants (12 females and 19 males) aged 21-70 years (mean 43.6 years). All were healthy members of the local community who consumed white wine at least once a month. Testing was performed in the sensory booths of the Cornell Food Research Lab, in Geneva, NY. The experimental procedure was reviewed and approved by the Cornell University Institutional Review Board. All participants provided written consent and were compensated for their participation.

The panelists executed a preference-ranking test (Lawless and Heymann, 2010). Subjects received a flight of four wines containing the control and three supplemented treatments for a given yeast strain, and were asked to rank them according to their preference (1=most preferred, 4=least preferred). In each session, three independent flights – one for each yeast strain tested –

were evaluated, for a total of 12 wines per session. The duplicate fermentations were pre-screened by chemical and sensory evaluation by nine panelists in the lab, and were considered to be the same sample for the purposes of sensory evaluation. In each flight, the order of samples was randomized for each participant. In the pre-screening, wines fermented by EC1118 and W15 were found to have low sugar-to-acid ratios, which gave them an excessively acidic taste. Since the focus of the study was to investigate differences in wine aroma, sugar adjustments were made by adding fructose, so that the four wines in a flight had the same sugar to acid ratio (Table 1.1).

Table 1.1: Sugar adjustments made to Riesling wines before sensory test

Yeast Strain	YAN Level	Replicate	Residual Sugar (g/L)	TA g/L	Initial Sugar: Acid Ratio	Target Sugar: Acid Ratio	Adjusted Sugar (g/L)
EC1118	130	1	0.16	8.21	0.02	0.5	4.11
	130	2	0.12	8.24	0.01		4.12
	180	1	0.24	8.78	0.03		4.39
	180	2	0.48	8.78	0.05		4.39
	250	1	0.41	8.92	0.05		4.46
	250	2	0.38	8.64	0.04		4.32
	300	1	0.38	9.20	0.04		4.60
	300	2	0.46	9.21	0.05		4.61
W15	130	1	3.56	9.32	0.38	1.0	9.32
	130	2	3.42	9.17	0.37		9.17
	180	1	2.80	9.20	0.30		9.20
	180	2	2.73	9.28	0.29		9.28
	250	1	4.44	9.69	0.46		9.69
	250	2	4.87	9.67	0.50		9.67
	300	1	4.94	9.72	0.51		9.72
	300	2	4.06	9.87	0.41		9.87

Statistical Analysis. Statistical analysis of wine composition parameters was performed using JMP version 11.2 (SAS Institute, Cary, NC). For wine non-volatile composition, separate one-way ANOVAs were conducted for each yeast strain, using YAN level as fixed effect. Where significant differences were encountered, comparison of means was made using Tukey-Kramer HSD test. Wine volatile compounds we analyzed using two-way ANOVAs, with YAN level and

yeast strain as fixed effects. For the eight fermentation products, linear regressions of relative response factors versus YAN level were made for each yeast strain using least squares. Statistical analysis for sensory data was made using tables for critical values of differences among rank sums (Basker, 1988).

Results

Model wine trials. The four yeast strains had different responses to DAP supplementation in synthetic media (Table 1.2). EC1118 was the most responsive to the different YAN levels. While it did not complete fermentation at 80 mg N/L, fermentation time was reduced from 17 to 12 days when initial YAN increased from 140 to 300 mg N/L. W15 and AWRI796 presented similar reductions. With 80 mg N/L, fermentation did not reach completion, leaving high levels of residual sugar – 39.6 and 26.7 g/L, respectively. With increasing YAN concentrations, both yeasts were able to ferment to dryness (defined as <2.0g/L residual sugars), reducing fermentation time from 19 to 15 days. Côte des Blancs was the only strain to ferment wines to dryness in the 80 mg N/L treatment. However, little reduction in fermentation time was observed in the remaining treatments, which ranged from 17 to 15 days. In all fermentations, YAN was completely exhausted from the media, except for Côte des Blancs at the 300 mg N/L level, where the final YAN concentration was 27 mg N/L (data not shown).

Table 1.2: Fermentation performance of four *S. cerevisiae* yeast strains in chemically defined grape juice (CDGJ) medium

Initial YAN (mg N /L)	Time to completion (days)				Residual sugar (g/L)			
	EC1118	W15	AWRI 796	Côte des Blancs	EC1118	W15	AWRI 796	Côte des Blancs
80	>20	>20	>20	20	7.2	39.6	26.7	<2
140	17	19	19	17	<2	<2	<2	<2
220	13	16	16	15	<2	<2	<2	<2
300	12	15	16	15	<2	<2	<2	<2

The media fermented by EC1118 had higher ethanol concentrations for the highest supplementation treatments (Table 1.4). With increasing YAN, a reduction in pH was observed, as well as an increase in lactic acid and decrease in malic acid concentrations (Tables 1.3 and 1.5). For W15 and AWRI796, ethanol also increased at higher YAN levels. Lactic acid concentrations also increased for both strains, though there was no impact on pH. Acetic acid concentration was higher at higher YAN levels for W15, but was not affected for AWRI796 (Table 1.4). Malic acid was unaffected in W15, and decreased in concentration for AWRI796. The wines fermented by Côte des Blancs did not differ in ethanol concentration or pH, but organic acid composition was dependent on initial YAN level. While acetic and lactic acid concentrations increased with increasing YAN, malic acid concentration was reduced. Given the similarities observed between W15 and AWRI796, the latter was not used in the subsequent fermentations conducted with Riesling grape juice.

Table 1.3: Mean values for pH and TA of synthetic wines produced by four *S. cerevisiae* strains

Initial YAN (mg/L)	pH				TA[1] (g/L)			
	EC 1118	W15	AWRI 796	Côte des Blancs	EC 1118	W15	AWRI 796	Côte des Blancs
80	3.21[a]	3.16	3.13	3.30[a]	6.60	6.88	7.51	6.52
140	3.17 [ab]	3.18	3.11	3.28[ab]	6.56	6.79	7.31	6.39
220	3.12[bc]	3.18	3.13	3.25[bc]	6.43	6.67	6.64	6.51
300	3.07[c]	3.16	3.10	3.23[c]	6.69	6.54	7.15	6.84
p-value	0.004	ns	ns	0.011	ns	ns	ns	ns

[1]*Expressed at Tartaric Acid Equivalents (TAE).*
Within a column, means followed by a different letter are significantly different using Tukey's test. $p < 0.05$ was considered significant.

Table 1.4: Mean values for ethanol and acetic acid of synthetic wines produced by four *S. cerevisiae* strains

Initial YAN (mg/L)	Ethanol (% v/v)				Acetic acid (g/L)			
	EC 1118	W15	AWRI 796	Côte des Blancs	EC 1118	W15	AWRI 796	Côte des Blancs
80	8.11^{c}	5.92^{c}	6.68^{b}	8.94	0.47	0.38^{b}	0.53	0.92^{b}
140	8.87^{ab}	8.10^{b}	8.26^{a}	8.84	0.50	0.51^{a}	0.58	0.99^{ab}
220	9.00^{a}	8.49^{ab}	8.30^{a}	8.54	0.54	0.48^{ab}	0.54	1.06^{ab}
300	8.37^{bc}	9.13^{a}	8.40^{a}	8.74	0.59	0.54^{a}	0.65	1.12^{a}
p-value	0.0068	0.0003	0.0002	ns	ns	0.0268	ns	0.0487

Within a column, means followed by a different letter are significantly different using Tukey's test. $p < 0.05$ was considered significant.

Table 1.5: Mean values for lactic and malic acids of synthetic wines produced by four *S. cerevisiae* strains

Initial YAN (mg/L)	Lactic acid (g/L)				Malic acid (g/l)			
	EC 1118	W15	AWRI 796	Côte des Blancs	EC 1118	W15	AWRI 796	Côte des Blancs
80	0.08^{d}	0.08^{c}	0.13^{b}	0.07^{d}	2.21^{a}	2.51	2.90^{a}	2.25^{a}
140	0.14^{c}	0.15^{b}	0.14^{ab}	0.14^{c}	2.16^{a}	2.79	2.79^{a}	2.13^{a}
220	0.20^{b}	0.21^{a}	0.20^{ab}	0.22^{b}	1.97^{ab}	2.36	2.32^{b}	1.95^{ab}
300	0.27^{a}	0.23^{a}	0.25^{a}	0.27^{a}	1.87^{b}	2.19	2.30^{b}	1.96^{ab}
p-value	<.0001	0.0002	0.0316	<.0001	0.0135	ns	0.0025	0.0025

Within a column, means followed by a different letter are significantly different using Tukey's test. $p < 0.05$ was considered significant.

Juice Chemistry. Juice titratable acidity (TA) as tartaric acid equivalents (TAE) was 7.98 ± 0.25g/L, and pH 3.14 ± 0.01. The YAN of the initial juice was 122 ± 1 mg N/L, and the unsupplemented control had a YAN concentration of 130 mg N/L after the addition of rehydration nutrient described above.

Fermentation rates and YAN concentration. The effect of nitrogen supplementation on fermentation kinetics was different for each yeast strain (Figure 1.1). EC1118 was able to ferment wines to dryness in all cases, even at the control concentration of 130 mg N/L. This strain was also the most responsive to DAP supplementation, decreasing fermentation time from

12 to 7 days at the highest nitrogen levels. However, there was no increase in fermentation rate when initial nitrogen was increased from 250 to 300 mg N/L.

Côte des Blancs was the least responsive to supplementation: fermentation time decreased only one day (from 15 to 14 days) as nitrogen concentration was increased from 130 to 180 mg N/L, and duration remained at 14 days for 250 and 300 mg N/L levels (Figure 1.1). Residual sugar levels decreased slightly as YAN increased: at 130 and 180 mg N/L, residual sugars averaged 2.23 g/L, while at 250 and 300 mg N/L, this average was 1.20 g/L. For W15, there was no increase in fermentation rates with nitrogen supplementation, and all wines had residual sugar levels > 2.0 g/L.

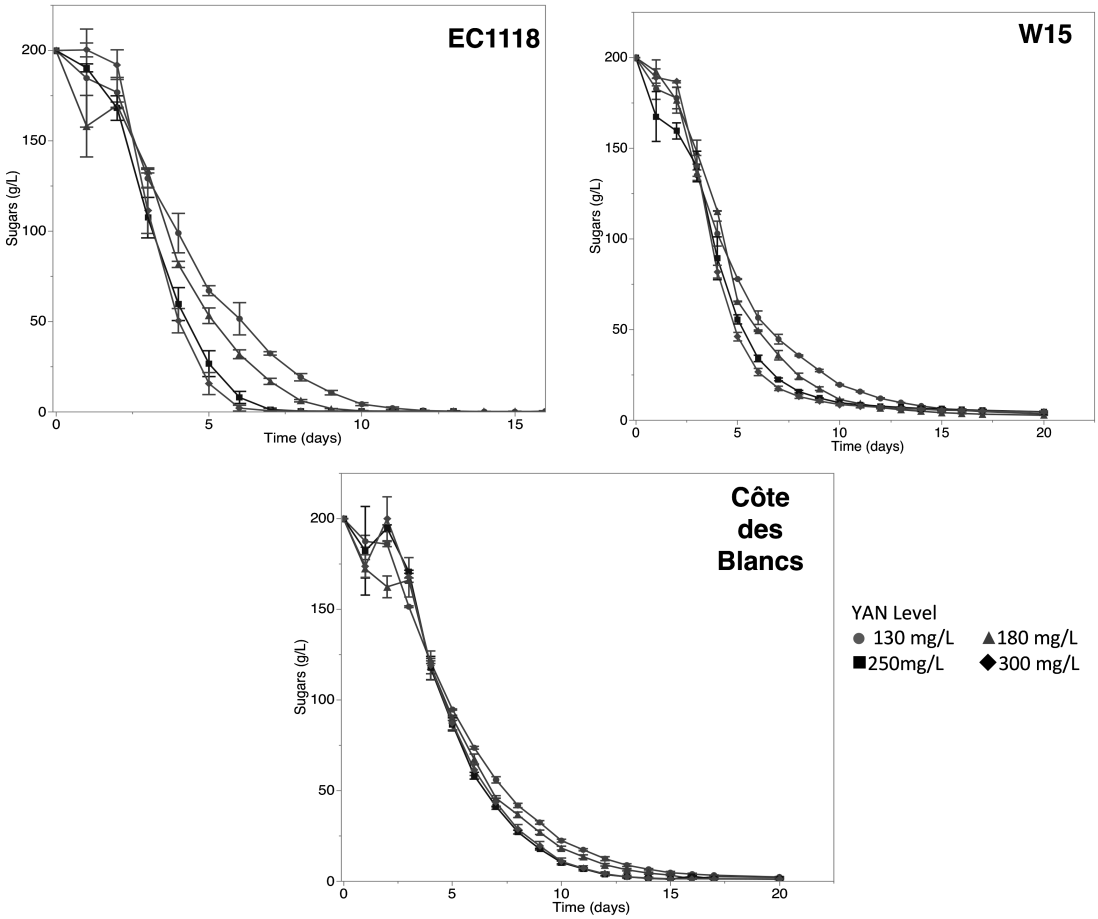

Figure 1.1: Consumption of total sugars during Riesling fermentation by three *S. cerevisiae* strains.

In all of the fermentations, AMM was consumed faster than PAN, but the consumption patterns of each YAN component varied by yeast strain (Figures 1.2 and 1.3). EC1118 exhausted all AMM present in the media within four days or less. On day 4, PAN was almost entirely consumed – from 93 to 99%, depending on the initial YAN level. After this point, all EC1118 fermentations had a rise in PAN, ranging from 11 to 21% of the initial PAN concentration. W15 showed similar nitrogen consumption, exhausting all AMM and PAN from the media at day 4. The increase in amino acid concentration at the later stages of fermentation also followed a similar pattern, except for the 300 mg N/L treatment, where there was a slightly larger increase. In that case, the wine showed a final YAN of 31 mg/L, and PAN at 34% of the initial concentration. In contrast, in Côte des Blancs fermentations AMM was only consumed entirely in the control and 180 mg N/L treatments. PAN was exhausted from the media only in the

control fermentations, but later increased to 19% of its initial concentration. This apparently low nitrogen uptake caused Côte des Blancs wines to have higher levels of residual YAN. While the control wines had a final YAN concentration of 17 mg N/L, YAN concentration increased to 66 and 105 mg N/L, respectively, in the 250 and 300 mg N/L treatments.

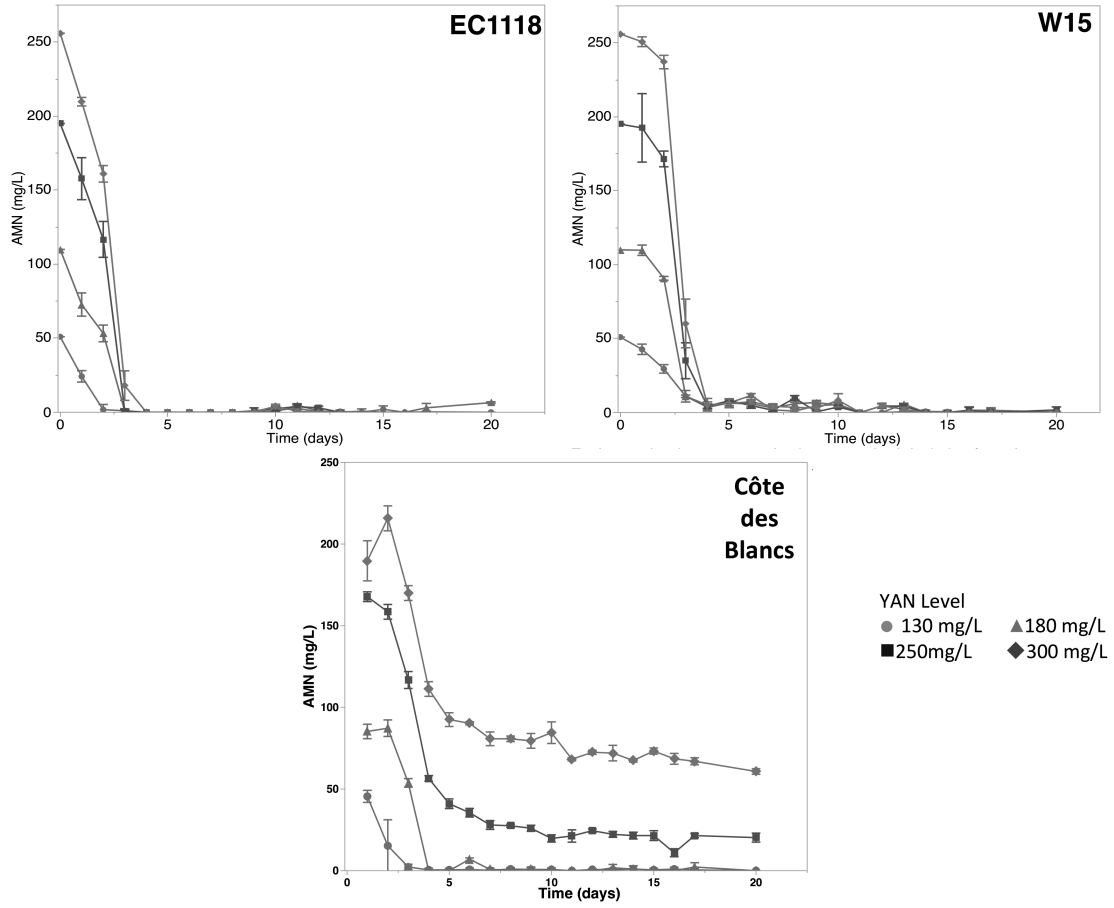

Figure 1.2: Consumption of ammonium during Riesling fermentation by three *S. cerevisiae* strains

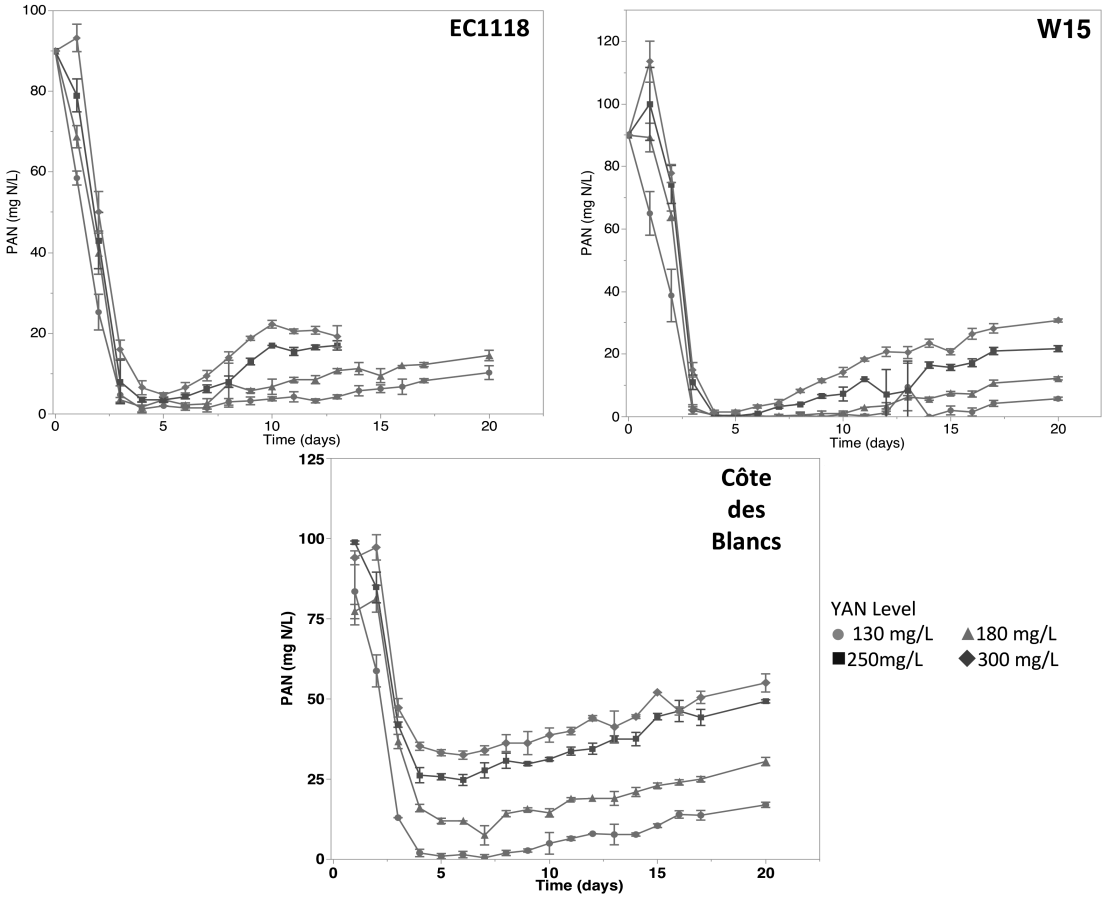

Figure 1.3: Consumption of primary amino nitrogen during Riesling fermentation by three *S. cerevisiae* strains

Final wine composition. EC1118. Fermentation rates for EC1118 were impacted by initial nitrogen concentration, but all wines finished with less then 0.5 g/L residual sugar (Table 1.6). Ethanol, pH and malic acid concentrations were unaffected by initial YAN (Tables 1.7 and 1.8). TA and lactic acid concentration increased with increasing YAN, while acetic acid concentration decreased.

W15. Residual sugar concentration varied among W15 treatments, although there was no clear trend (Table 1.6). Ethanol production was not affected by YAN. There was a decrease in pH and increase in TA with higher YAN supplementation levels, and concentrations of organic

acids were affected as well; final malic acid and acetic acid concentration decreased with increased YAN supplementation, while the production of lactic increased (Tables 1.7 and 1.8).

Côte des Blancs. The 250 and 300 mg/L Côte des Blancs treatments were notably lower in residual sugar than the control and 180 mg N/L treatment; ethanol concentration, however, was not affected (Table 1.6). At higher concentrations of initial YAN, there was an increase in pH, TA and malic acid were not significantly affected, but lactic and acetic acids increased with YAN supplementation (Tables 1.7 and 1.8).

In summary, although initial YAN concentrations affected residual sugar for two of the three yeast strains studied, ethanol concentration was not affected in any case. There was a trend of increasing TA as the DAP supplementation increased, and this effect was significant for two of the yeast strains. For all three strains, production of lactic acid increased with higher initial YAN concentrations. Production of acetic acid was also affected, although the initial YAN concentration that favored maximum production varied with each yeast strain.

Table 1.6: Mean values of ethanol and residual sugar levels of Riesling wines produced with three *S. cerevisiae* yeast strains.

	Ethanol (%v/v)			Residual Sugar (g/L)		
Initial YAN (mg N/L)	EC1118	W15	Côte des Blancs	EC1118	W15	Côte des Blancs
130	11.70	11.59	11.72	0.14	3.49^{ab}	2.35^{a}
180	11.31	11.69	11.78	0.36	2.77^{b}	2.12^{ab}
250	11.82	11.57	11.82	0.40	4.66^{a}	1.16^{b}
300	11.79	11.44	12.18	0.42	4.50^{a}	1.24^{b}
p-value	n.s	n.s	n.s	n.s	0.0160	0.0204

Within a column, means followed by a different letter are significantly different using Tukey's test. $p < 0.05$ was considered significant.

Table 1.7: Mean values for pH and TA of Riesling wines produced with three *S. cerevisiae* yeast strains.

	pH			TA[1] (g/l)		
Initial YAN (mg N/L)	EC1118	W15	Côte des Blancs	EC1118	W15	Côte des Blancs
130	3.04	3.12^{a}	3.07^{ab}	8.23^{c}	9.25^{b}	8.31
180	3.03	3.11^{ab}	3.07^{b}	8.78^{b}	9.24^{b}	8.48
250	2.99	3.09^{ab}	3.06^{b}	8.78^{b}	9.68^{a}	8.57
300	2.97	3.08^{b}	3.10^{a}	9.21^{a}	9.80^{a}	8.54
p-value	n.s	0.0431	0.0235	0.0029	0.0044	n.s

[1] *Expressed at Tartaric Acid Equivalents (TAE)*
Within a column, means followed by a different letter are significantly different using Tukey's test. $p < 0.05$ was considered significant.

Table 1.8: Mean values for organic acids composition of Riesling wines produced with three *S. cerevisiae* yeast strains.

	Malic Acid (g/L)			Lactic Acid (g/L)			Acetic Acid (g/L)		
Initial YAN (mg N/L)	EC1118	W15	Côte des Blancs	EC1118	W15	Côte des Blancs	EC1118	W15	Côte des Blancs
130	3.04	3.32^{a}	2.88	0.22^{d}	0.29^{c}	0.22^{c}	0.34^{a}	0.25^{a}	0.58^{b}
180	3.04	3.17^{b}	2.90	0.32^{c}	0.32^{bc}	0.27^{b}	0.26^{ab}	0.18^{b}	0.59^{ab}
250	3.03	3.14^{bc}	2.87	0.41^{b}	0.39^{ab}	0.30^{a}	0.18^{b}	0.15^{c}	0.64^{a}
300	3.04	3.07^{c}	2.87	0.47^{a}	0.44^{a}	0.28^{b}	0.18^{b}	0.15^{c}	0.61^{ab}
p-value	n.s	0.002	n.s	<.0001	0.0052	<.0001	0.0146	<.0001	0.031

Within a column, means followed by a different letter are significantly different using Tukey's test. $p < 0.05$ was considered significant.

Concentration of volatile compounds. Eight of the ten volatile compounds analyzed via GC-MS were affected by initial nitrogen concentration for at least one of the yeast strains (Table 1.10). In the two-way ANOVA, a significant interaction was found for YAN level x yeast strain for seven of those compounds, suggesting that their synthesis is dependent on both the yeast strain and initial YAN level of the juice. In general, ester concentration increased with higher

nitrogen supplementation (Figures 1.4 and 1.5). One compound to note is 3-methyl butyl acetate, which showed increased concentrations at higher nitrogen levels across all yeast strains. Ethyl hexanoate was only significantly impacted for W15, where concentrations increased with more DAP supplementation. Ethyl octanoate was impacted for EC1118 and Côte des Blancs, where supplementation treatments had higher ester concentrations than the control. Impacts on ethyl propanoate production varied with yeast strain; for EC1118, the fermentations with 300 mg/L initial YAN had a significantly higher concentration than the other treatments. For Côte des Blancs, the 250 mg/L initial YAN was the concentration that favored maximum production, and no change was seen in W15 fermentations. Ethyl cinnamate showed an opposite trend, decreasing in concentration at higher nitrogen levels in W15 and Côte des Blancs fermentations.

Table 1.9: Retention times of volatiles analyzed in Riesling wines via GC-MS.

	Retention Time	
	(min)	**m/z**
Ethyl propanoate	3.60	29,57, 27
1-Hexanol	7.53	56, 43, 69, 84
3-Methyl butyl acetate	7.74	43, 70, 55, 87
Ethyl hexanoate	11.35	88, 99, 60, 144
Linalool	14.30	71, 93, 121, 154
2-Phenylethanol	15.59	91, 122, 65, 51
Ethyl octanoate	16.83	88, 101, 127, 172
1,1,6-Trimethyl-1,2-dihydronaphthalene (TDN)	20.80	157, 142, 172
Decanoic acid	21.74	88, 101, 73, 60
Ethyl cinnamate	23.47	131, 103, 176, 77
2-octanol (internal standard)	11.40	45, 97, 70

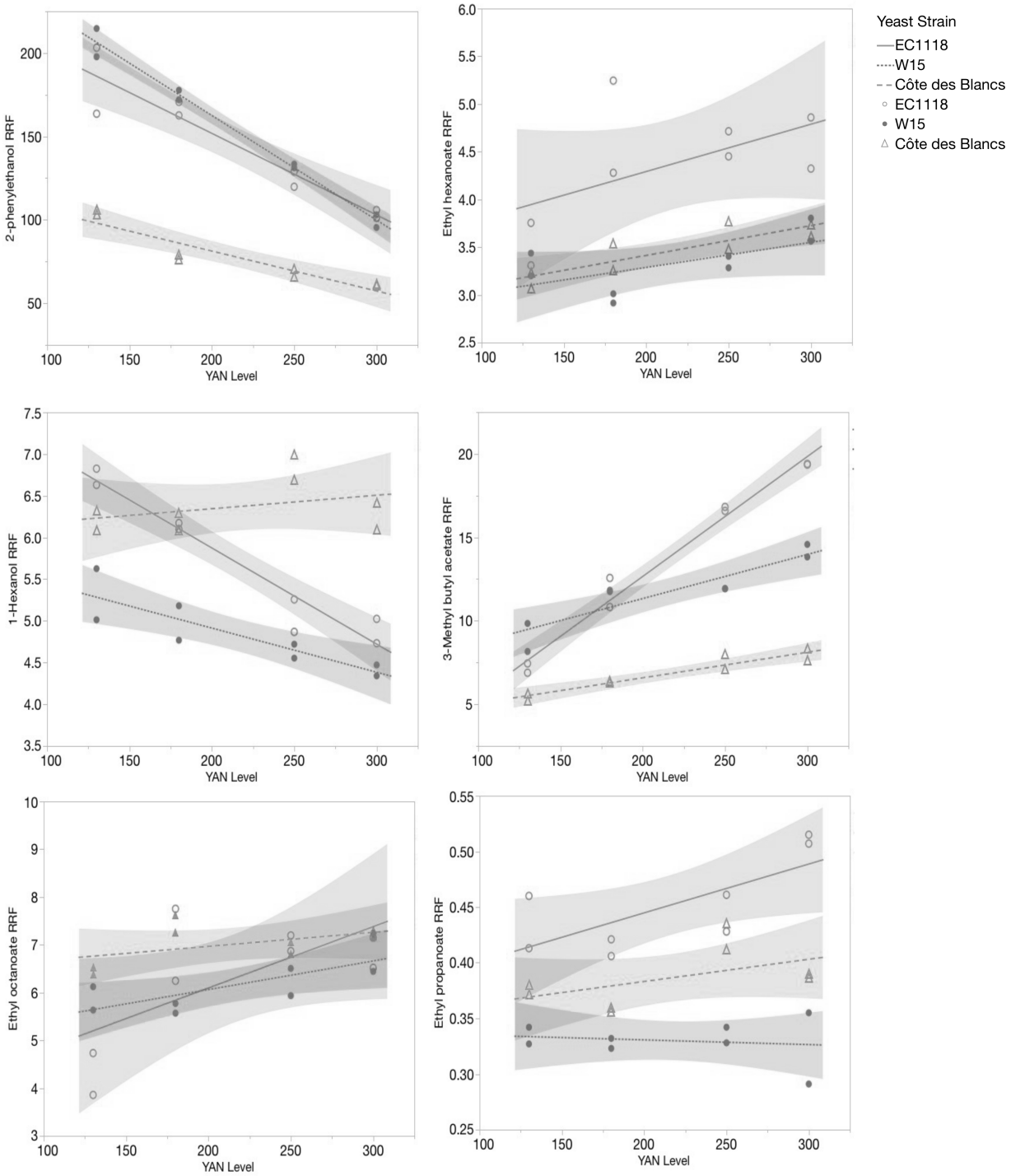

Figure 1.4: Linear fit of volatile fermentation product RRFs as a function of initial YAN concentration. Shaded areas represent the 95% confidence interval for the regression parameters. RRF = relative response factor. YAN=Yeast Assimilable Nitrogen.

Of the two higher alcohols analyzed in this study, 2-phenylethanol consistently decreased with DAP addition. In contrast, 1-hexanol showed a decrease in concentration only in EC1118 fermentations. For the wines fermented with EC1118, all supplemented treatments had higher decanoic acid concentrations than the unsupplemented control. Wines fermented with Côte des Blancs had an increase in decanoic acid concentration with 180 mg/L initial YAN level, but the other treatments and the control were not different.

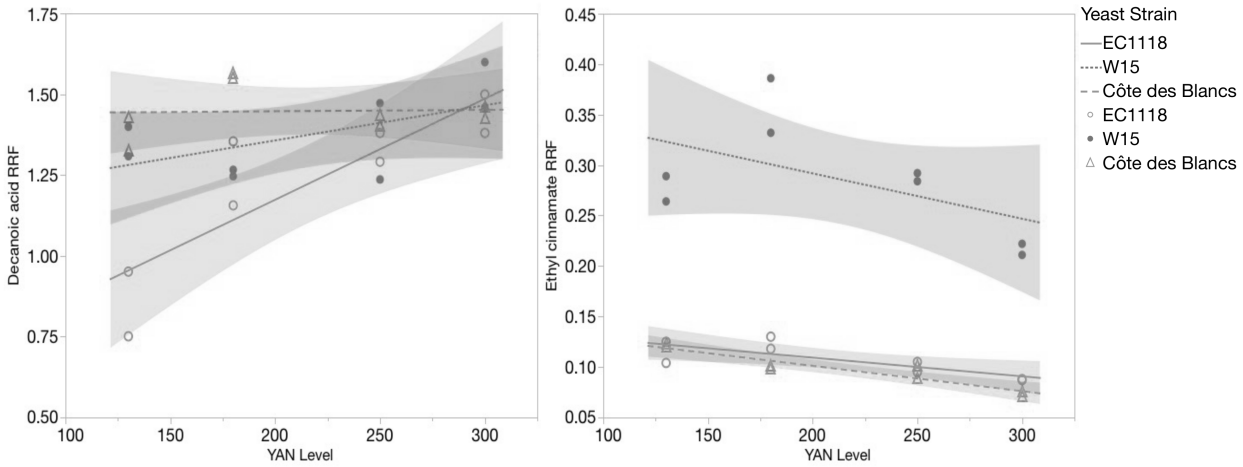

Figure 1.5: Linear fit of volatile fermentation product RRFs as a function of initial YAN concentration. Shaded areas represent the 95% confidence interval for the regression parameters. RRF = relative response factor. YAN = Yeast Assimilable Nitrogen.

Linear regression models were run for the RRF's of the volatile fermentation products as a function of initial YAN concentration, and for some cases the model accurately represented the relationship between the data (Tables 1.11 and 1.12). For instance, 2-phenyl ethanol had different rates of decrease for Côte des Blancs, but fairly similar rates for EC1118 and W15, as can be seen by comparing the slopes and confidence intervals (Figure 1.4). For 3-methyl butyl acetate, the rates of increase with increasing YAN were different for each yeast strain.

Table 1.10: p-values for volatile fermentation products obtained by 2-way ANOVA considering the effects of yeast strain and initial YAN[1] level.

Compound	Effects		
	Yeast Strain	YAN level	Yeast Strain x YAN level
2-phenylethanol	<0.0001*	<0.0001*	0.0065*
Ethyl hexanoate	<0.0001*	0.0087*	0.0523
1-Hexanol	<0.0001*	<0.0001*	0.0002*
3-methyl butyl acetate	<0.0001*	<0.0001*	<0.0001*
Ethyl Octanoate	0.0058*	0.0007*	0.0108*
Ethyl Propanoate	<0.0001*	0.0116*	0.0177*
Decanoic Acid	0.0010*	0.0017*	0.0057*
Ethyl Cinnamate	<0.0001*	<0.0001*	0.0009*

[1]Yeast assimilable nitrogen

*Significance was found using $\alpha = 0.05$

Table 1.11: Linear regression model coefficients and coefficient of determination (R^2) for volatile fermentation products as a function of YAN[1] in wines fermented by EC1118 and W15.

Compound	EC1118			W15		
	Slope	Intercept	R^2	Slope	Intercept	R^2
2-phenylethanol	-0.4891±0.0686	249.66±15.403	0.8945	-0.6265±0.0309	287.86±6.943	0.9856
Ethyl hexanoate	0.0050±0.0030	3.3010±0.6719	0.3140	0.00260.0013	2.7618±0.2976	0.3957
1-Hexanol	-0.0115±0.0012	8.1797±0.2759	0.9362	-0.0053±0.0012	5.9720±0.2748	0.7572
3-methyl butyl acetate	0.0718±0.0041	-1.7014±0.9227	0.9807	0.0264±0.0051	6.0478±1.1433	0.8179
Ethyl Octanoate	0.0128±0.0058	3.5309±1.3068	0.4478	0.0060±0.0022	4.8652±0.4946	0.5519
Ethyl Propanoate	0.0004±0.0002	0.3570±0.0380	0.5289	-0.0000±0.0001	0.3391±0.0246	0.0242
Decanoic Acid	0.0031±0.0008	0.5480±0.1714	0.7368	0.0011±0.0006	1.1397±0.1408	0.3333
Ethyl Cinnamate	-0.0002±0.0001	0.1464±0.0134	0.6154	-0.0004±0.0003	0.3815±0.0622	0.3047

[1] Yeast Assimilable Nitrogen

The average TDN concentration in the final wines was 2.7 μg/L, and there was no effect of yeast strain or initial YAN level (Table 1.13). According to the two-way ANOVA, linalool concentrations in the final wines did not depend on the initial YAN level of the juice (p-value = 0.5813), but were dependent on yeast strain (p-value=0.0052). Côte des Blancs wines had a final linalool concentration of 36.9 ±3.0 μg/L, higher than that of the wines fermented with EC1118 and W15 at 33.9±1.5 μg/L and 33.2 ±SD 1.1μg/L, respectively.

Table 1.12: Linear regression model coefficients and coefficient of determination (R^2) for volatile fermentation products as a function of YAN[1] in wines fermented by Côte des Blancs.

Compound	Côte des Blancs		
	Slope	Intercept	R^2
2-phenylethanol	-0.2393±0.0369	129.15±8.281	0.8754
Ethyl hexanoate	0.0031±0.0008	2.7884±0.1755	0.7268
1-Hexanol	0.0016±0.0018	6.0218±0.4026	0.1191
3-methyl butyl acetate	0.0153±0.0021	3.5207±0.4746	0.8974
Ethyl Octanoate	0.0029±0.0022	6.3797±0.4847	0.2360
Ethyl Propanoate	0.0002±0.0001	0.3432±0.0301	0.2701
Decanoic Acid	0.0000±0.0005	1.4387±0.1027	0.0014
Ethyl Cinnamate	-0.0003±0.0000	0.1513±0.0085	0.8789

[1] Yeast Assimilable Nitrogen

Table 1.13: TDN[1] concentrations of Riesling wines produced from juices with various YAN[2] levels

	TDN (μg of naphthalene/L)		
Initial YAN (mg N/L)	EC1118	W15	Côte des Blancs
130	2.653	2.652	2.652
180	2.654	2.652	2.651
250	2.655	2.652	2.653
300	2.655	2.652	2.651
p-value	n.s	n.s	n.s

[1]TDN: 1,1,6-Trimethyl-1,2-dihydronaphthalene

[2]YAN: yeast assimilable nitrogen

Sensory evaluation. A difference in preference was found only in wines fermented with EC1118 (Table 1.14). The control wine was most preferred overall, followed by 180 and 250 mg/L treatments, which were ranked equally. The 300 mg/L treatment was least preferred.

Table 1.14: Sum of preference ranking scores for Riesling wines fermenting at different YAN levels with different *S. cerevisiae* strains.

Initial YAN (mg N/L)	EC1118	W15	Cote des Blancs
130	62^{c}	62^{a}	72^{a}
180	73^{bc}	83^{a}	74^{a}
250	74^{bc}	83^{a}	85^{a}
300	101^{a}	82^{a}	79^{a}

Scores with different superscripts indicate that the preference for this treatment significantly differs from the others at the significance level $p<0.05$.

Discussion

Nitrogen Consumption. YAN consumption was directly proportional to initial YAN concentration and strongly dependent on yeast strain, as observed in similar studies (Vilanova et al. 2007, Ugliano et al. 20011). For the control fermentations, nitrogen consumption ranged from 113 mg N/L (Côte des Blancs) to 124 mg N/L (W15). As the initial YAN increased, all the three strains increased consumption; EC1118 had the highest uptake, consuming 280 mg N/L in the 300 mg N/L supplementation treatment. Meanwhile, Côte des Blancs had the lowest consumption, and the nitrogen uptake peaked at 195 mg/L for the highest supplementation treatment. It is known that AMM ions are the preferred source of nitrogen by yeasts, and are consumed faster than amino acids (Miller et al. 2007). In this work, AMM was observed to be completely exhausted from the media in a few days in most of the fermentations (Figure 1.2). In contrast, PAN was either exhausted or reached a minimum (between 3.5 and 32.5 mg N/L) before the trend reversed and concentrations increased to reach a fraction of the initial value by the end of fermentation (Figure 1.3). Such nitrogen release is expected in the late stages of fermentation, and is attributed to a higher membrane permeability caused by a high ethanol

concentration (Salgueiro et al. 1988). As the yeast population enters the death phase, amino acids continue to be liberated in the media by passive diffusion from the intercellular pool through the first stage of yeast autolysis (Alexandre et al. 2001).

Minimum YAN concentration. YAN supplementation impacted fermentation kinetics for EC1118 most, reducing fermentation time by 5 days within the supplementation range studied (130 to 300 mg N/L). W15 fermentations times did not change, and Côte des Blancs supplementation treatments fermented one day faster than the control. Although there were differences in residual sugar for W15 and Côte des Blancs, ethanol concentration was not affected by YAN for any of the yeast strains (Table 1.6). This suggests that the YAN concentration of the control juice (130 mg N/L) was sufficient to complete fermentation for the three yeast strains studied. This finding is in line with the accepted literature, which often considers 140 mg/L as the minimum YAN required to complete alcoholic fermentation in a 20°Brix must (Butzke 1998, Bell and Henschke 2005). Since 20°Brix is a typical level of harvest soluble solids in cool-climate regions (Nisbet et al. 2014), the recommendation of 140 mg N/L could be generally applied. For musts with higher sugar levels, Bisson and Butzke (2000) recommended an additional 25 mg N/L of YAN for every 1°Brix increase. However, it has been shown that fermentation completion did not depend on this additional YAN supplementation, even for musts of higher sugar levels (Childs et al. 2015, Bely et al. 2003). The addition of nitrogen could still have benefits for wine quality, however, by changing volatile composition.

Fermentation performance of different yeast strains. Bisson and Butkze (2000) note that there are basically two types of yeast fermentation profiles. Strains with a *S. bayanus* background, such as EC1118, are typically strong fermenters displaying short lag phases and high fermentation rates. In contrast, Côte des Blancs and related yeasts are characterized as slow

fermenters. Several traits influence a yeast's fermentation performance, and nitrogen metabolism seems to be one of the most important (Treu et al. 2014). EC1118 has high expression of genes regulating nitrogen uptake, and this is likely responsible for its high fermentation speed (Treu et al. 2014). This is consistent with the current study, where EC1118 had the shortest fermentation times and the highest nitrogen consumption. Nevertheless, it was able to ferment all wines to dryness, even the unsupplemented control. Though there are no studies linking gene expression to nitrogen uptake in W15, the high nitrogen consumption agrees with previous literature showing that this strain has a high nitrogen demand (Vilanova et al. 2007). In contrast, a previous experiment that used synthetic grape juice medium (Vilanova et al. 2007), showed no gain in fermentation speed with nitrogen supplementation. In a study conducted with common commercial strains, Côte des Blancs was both a slow fermenter and was notably the least active in terms of amino acid uptake (Ough et al. 1991). This is similar to the present work, as Côte des Blancs fermentations had the highest levels of residual YAN.

Changes in non-volatile composition of wines. DAP additions affect the balance of organic acids and the final amino acid concentration in the wines, often leading to a higher TA and lower pH (Ugliano et al. 2007, Torrea et al. 2011, Vilanova et al. 2012). In this study, TA tended to increase with higher DAP additions, but pH was only marginally affected. The production of acetic acid decreased between control and treatment fermentations for EC1118 and W15, but increased for Côte des Blancs (Table 1.8). It has been suggested that one of the drivers of acetate production during alcoholic fermentation is the regeneration of NADH to maintain cell redox balance in a high osmolar medium (Bely et al. 2003). Increased nitrogen availability increases NADH production, which in turn reduces the need for acetate production. An excess of nitrogen compounds, however, can increase the production of volatile acidity, as seen with Côte

des Blancs. This effect has been observed in other studies, but the mechanisms behind it are still unclear (Bely et al. 2003, Hernandez-Orte et al. 2006, Vilanova et al. 2007).

Production of volatile compounds. Although 140 mg N/L is generally recognized as the minimum YAN concentration for fermentation completion, several studies suggest that supplementation to final levels as high as 260-500 mg N/L can positively impact wine aroma by increasing ester production and decreasing production of higher alcohols and hydrogen sulfide (Vilanova et al. 2012, Torrea et al. 2011, Miller et al. 2007, Ugliano et al. 2011). This was confirmed in the present study, where most of the esters measured were found in higher concentrations in supplemented fermentations. Of the two higher alcohols quantified, 2-phenylethanol decreased with higher DAP in all yeast strains, and 1-hexanol decreased in EC1118, though concentrations were generally lower (though not significantly so) across yeast strains (Figure 1.4).

There are two means by which nitrogen could affect the final monoterpene concentration of wines. The first is by changing the activity of must glycosidases, therefore increasing the hydrolysis of grape precursors (Bell and Henschke 2005). The second, and most likely, is by influencing the *de novo* synthesis of monoterpenes through a defect in the sterol pathway (Swiegers et al. 2005). In this study, no influence from nitrogen supplementation was observed in the final linalool concentration. Nevertheless, the maximum YAN concentration used in our study was 300 mg N/L, while in studies reporting an increase in terpene production, supplementation levels reached as high as 450 mg N/L (Carrau et al. 2005, Vilanova et al. 2012). Glycosidase activity can likewise affect the production of TDN precursors, but no differences were found in TDN concentrations among fermentation treatments. Formation of TDN in wines involves multiple transformations after the initial hydrolysis; thus, TDN tends to appear during

bottle storage. Since GC analysis was performed only 3 months after bottling, it is possible that TDN concentrations had not fully developed.

Sensory evaluation. All wines showed differences in volatile and non-volatile composition following DAP additions, but sensory differences among the treatments were only detected in the wines fermented by EC1118. In that case, the control wine was most preferred by the taste panel, followed by the 180 and 250 mg N/L, with 300 mg N/L treatment least preferred. This preference ranking may be due to the increase in acetate esters (Figure 1.4), which might confer nail polish or bruised apple aromas, as observed previously (Ugliano et al. 2010, Torrea et al. 2011). Other changes in volatile composition that might have contributed to the lower preference for supplemented wines include the measured decrease in concentration of the alcohol 2-phenylethanol, which has a flowery and rose-like odor (Komes and Lovric 2005). Ethyl cinnamate, which possesses a fruity odor (Bowen and Reynolds 2012), also decreased in concentration in the supplementation treatments. Additionally, production of decanoic acid, which has a rancid and unpleasant odor (Gil et al. 2006), increased with DAP addition. Since there was also no increase in the concentration of varietal aromas such as linalool and TDN, this data suggests no evident benefit of DAP supplementation to the aroma expression of Riesling wine.

Conclusion

This study found that a YAN concentration of 130 mg N/L is enough to complete fermentation of a 20°Brix must for the three yeast strains used, though DAP supplementation up to 250 mg N/L improved fermentation kinetics for EC1118. Fermentation speed of Côte des Blancs and W15 did not increase with DAP supplementation. YAN consumption was strain-

dependent, and increased with nitrogen supplementation, ranging from 113 to 281 mg N/L. Côte des Blancs had the lowest YAN consumption, and the 300 mg N/L treatment ended with 105 mg N/L of residual YAN. This suggests that 300 g N/L is an excessive initial YAN concentration for Côte des Blancs.

YAN concentrations ranging from 130 to 300 mg/L, obtained with DAP additions, showed significant effects in wine volatile and non-volatile composition. Two varietal compounds (TDN and linalool) were quantified, and their concentrations were found to be unrelated to YAN concentration. While relative concentrations of most esters analyzed increased in the supplementation treatments, sensory panelists either showed no clear preference among treatments (for W15 and Côte des Blancs), or preferred the unsupplemented wine (for EC1118). This conflicts with previous literature suggesting that YAN concentrations higher than 140 mg N/L improve volatile composition and wine quality. Considering the moderate sugar levels common to musts of Finger Lakes Riesling, winemakers must avoid over-addition of nitrogen, which might generate microbial instability and a decrease in desired sensory properties.

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CHAPTER 2
CONSUMPTION OF YAN DURING SPONTANEOUS FERMENTATIONS IN THE FINGER LAKES

Abstract: Spontaneous fermentations are a growing trend in winemaking, yet little work has been done to characterize them in the Finger Lakes region. Moreover, microbial ecology of spontaneous fermentations can vary greatly across different wine regions. The objective of this study, performed in collaboration with two local wineries, was to provide an initial assessment of microbial diversity and nitrogen requirements of such fermentations. During the 2014 harvest, five single-vineyard Riesling fermentations were monitored. Prior to harvest, grape samples were collected to analyze microflora present on the surface of the berries. Winery equipment was also sampled to assess winery microflora. The initial juices ranged between 17-20 °Brix, and YAN, residual sugar, and microbial count were monitored from samples taken at each 5 °Brix reduction throughout fermentation. Preliminary microbe identification was made by colony morphology, and representative samples were isolated and analyzed by qPCR and VNTR (Variable Number of Tandem Repeats). Non-*Saccharomyces* yeasts persisted until the last stages of fermentation for all five vineyards, although always in smaller numbers than *S. cerevisiae*. The most frequent non-*Saccharomyces* yeasts found were *Hanseniaspora uvarum*, *Pichia fermentans* and various *Kluyveromyces* spp. The two wineries differed in microflora composition, with one exhibiting a larger diversity of non-*Saccharomyces* yeasts, while the other had a larger number of *S. cerevisiae* strains isolated. The diversity of *S. cerevisiae* strains isolated suggests that no single strain was dominant, though several were involved in the fermentation. Long fermentation times were observed,

ranging between 18-98 days, and in one winery lag phases were also lengthy (27 days on average). YAN consumption ranged from 59 to 166mg/L among fermentations, and results suggest that, for the vineyards monitored, spontaneous fermentations do not have higher YAN requirements than inoculated fermentations.

Introduction

The fermentation of grape juice into wine is a complex microbial reaction, usually involving a sequential development of different species of yeast and bacteria (Fugelsang and Edwards 2007). In modern winemaking, the use of commercial yeast starter cultures is a common practice, given the higher predictability that they provide (Pretorius 2000). However, conducting spontaneous or non-inoculated fermentations has been a growing trend, and is becoming more common in the Finger Lakes region of New York.

The yeasts that arise in spontaneous fermentations can be broadly divided into two groups: *Saccharomyces cerevisiae* and the non-*Saccharomyces* yeasts. *S. cerevisiae* can originate from the vineyard, resident winery microflora, or from commercially produced strains, and evidence suggests that gene flow occurs relatively frequently across those different populations (Hyma and Fay 2013, Knight and Goddard 2015). Non-*saccharomyces* yeasts are usually indigenous to the vineyard and grape environment, and the most common species include *Kloeckera apiculata*/*Hanseniaspora uvarum*, *Candida*, *Cryptococcus*, *Debaryomyces*, *Hansenula*, *Issatchenkia*, *Kluyveromyces*, *Metschnikowia*, *Pichia*, and *Rhodotorula* (Fugelsang and Edwards 2007). On grape berries, the microflora composition is dependent on many factors, including physical damage or mold, rainfall, and use of pesticides. In wineries, the use of sulfur dioxide, cleaning and

sanitization programs, and degree of juice clarification all affect the microbial ecology of fermentation. (Longo et al. 1991, Pretorius 2000, Hierro et al. 2006).

Differences in the composition of fermentation microflora have been reported to impact flavor profile of finished wines, a concept that has been called "microbial terroir" (Medina et al. 2013, Knight et al. 2015). For instance, spontaneously fermented wines are generally regarded as having more desirable sensory properties, such as more aromatic complexity and better mouthfeel. These changes can be attributed to the tendency of non-saccharomyces species to secrete a larger number of enzymes (esterases, glycosidases, β-glucosidases, proteases, etc.) that are likely to increase production of aroma compounds (Izquierdo Cañas 2011). Given the variability that spontaneous fermentations show across different regions, and the potential impacts on wine quality, several studies have focused on characterizing the microbial ecology of those fermentations in different winemaking regions (Granchi et al. 2003, Combina et al. 2005, Hall et al. 2011, Csoma et al. 2010). However, the only report on spontaneous fermentations from the Finger Lakes region of New York is a study focusing on *Hanseniaspora* spp. characterization (Bujdosó et al. 2001).

The growth of non-*Saccharomyces* yeasts can have inhibitory effects on *S. cerevisiae*, which is the most efficient fermenter (Boulton et al. 1999). This competition poses a risk for spontaneous fermentations to become stuck or sluggish. Limitations of nutrients, especially nitrogen, are among the causes for this phenomenon, but nitrogen requirements of spontaneous or mixed culture fermentations have not been extensively reported in literature (Medina et al. 2012, Barrajón-Simancas et al. 2011). On the other hand, the sequential growth of different species and strains on a spontaneous

fermentation can also be beneficial. For instance, the early death and autolysis of some non-*Saccharomyces* yeasts, as well as the proteolytic action of some species, is a possible source of nutrients for *S. cerevisiae* (Fleet 2003).

Although a variety of nitrogen compounds are found in grapes, only some of them can be assimilated by yeast during fermentation. This usable nitrogen fraction is referred to as Yeast Assimilable Nitrogen (YAN), and is defined as the sum of the ammonia (AMM) and primary amino nitrogen (PAN) concentrations (Monteiro and Bisson 1991, Henschke and Jiranek 1993). Proteins, peptides, and secondary amino acids such as proline cannot be metabolized, and are excluded from YAN measurements. In an initial assessment of YAN requirements and microbiome, five spontaneous fermentations in two commercial Finger Lakes wineries were monitored during the 2014-2015 season. Fermentation kinetics, YAN consumption, yeast and bacteria were characterized in an effort to better understand the interaction of microflora and nitrogen requirements in spontaneous fermentations.

Materials and Methods

Wines. The five commercial wines studied were single-vineyard Rieslings produced in two Finger Lakes wineries. Fermentations A, B and C took place in winery 1, and fermentations D and E in winery 2. In all cases, the grapes were whole-cluster pressed and received an SO_2 addition according to each winery's protocol (35 mg/L in Winery 1, and 30 mg/L in Winery 2). Following pressing, juice was transferred into stainless steel clarification tanks, settled for 24 to 48h, then racked into stainless steel fermentation tanks. The chemical composition of the five juice lots is shown in Table 2.1.

Table 2.1: Chemical composition of Riesling grape juice from five different vineyards

Parameter	Vineyard[1]				
	A	B	C	D	E
Sugar concentration (g/L)	180	199	182	183	166
pH	3.16	3.15	3.21	3.18	3.00
Titratable acidity	7.7	7.5	7.3	10.1	9.0
YAN (mg N/L)	119	105	144	231	68

[1]Vineyards A, B and C: Winery 1; vineyards D and E: Winery 2.

Sampling. Before harvest**,** grape samples were collected from each vineyard (Table 2.2). A total of 10 clusters were collected across the central row of each vineyard block, using aseptic methods, and placed in plastic Ziploc™ bags. The grapes were crushed by hand within the bag to obtain a juice aliquot for microbial analysis. The surface of winery equipment (press, pumps, clarification and fermentation tanks) was sampled following a procedure described elsewhere (Mercado et al. 2007). In short, samples were taken by streaking approximately 300cm^2 of each surface with sterile cotton plugs. The sorting table, press and pump were sampled during use, and clarification and fermentation tanks were sampled when they were clean, sanitized and ready to receive the juice.

Duplicate 25mL wine samples were taken at frequent intervals during vinification. Once the juice was transferred into the fermentation tank, samples were taken 3 times a week for up to 2 weeks. In cases where fermentation did not start after two weeks, sampling was paused and resumed at the beginning of fermentation. After fermentation commenced, samples were taken at approximately every 5° Brix reduction. Fermentation samples underwent microbial enumeration and isolation as described below, and YAN and sugar concentrations were quantified for all samples.

Table 2.2: Dates of initial sampling events for five different Riesling vineyards

	Vineyard				
	A	B	C	D	E
Grape sampling	7 Oct 2014	7 Oct 2014	7 Oct 2014	10 Oct 2014	11 Oct 2014
Harvest	20 Oct 2014	22 Oct 2014	24 Oct 2014	11 Oct 2014	11 Oct 2014
Equipment swabs	20 Oct 2014	20 Oct 2014	20 Oct 2014	13 Oct 2014	13 Oct 2014
Juice pressing	20 Oct 2014	22 Oct 2014	24 Oct 2014	13 Oct 2014	13 Oct 2014

Microbial Enumeration and Isolation. For all samples (grapes, equipment swabs and fermentation), a dilution series from 10^0 to 10^{-7} was prepared. Dilutions were plated, in duplicate, in Wallerstein Laboratory (WL) Nutrient Agar (Sigma-Aldrich, St Louis, MO), and WL agar with addition of 100 mg/L of cycloheximide to select for growth of bacteria over yeasts. WL medium allows presumptive discrimination between the yeast species by colony morphology and color (Pallman et al. 2001). Fermentation samples were also plated in Lysine agar as described in Martin and Siebert (1992). Plates were incubated at 18°C for 7 days, after which various colony types were counted and representative colonies were isolated and subcultured.

Microbial Identification. Representative colonies of each morphology were subcultured in WL agar and incubated at 18°C for 7 days. After incubation, the plates were shipped overnight to ETS Laboratories (St. Helena, CA) for molecular identification of yeasts and bacteria. An initial screening was performed via qPCR utilizing the Scorpions Wine Spoilage Systems module, as described elsewhere (Umiker et al. 2012). The Scorpions system is able to identify yeasts and bacteria species that are most commonly present on wine fermentations.

The isolates identified as *S. cerevisiae* and those that could not be identified via Scorpions were submitted to Variable Number of Tandem Repeats (VNTR) analysis. The method targeted five loci specific to *S. cerevisiae*: SC8132X, YOR267C, C5, C11 and C12b. Additionally, a primer set amplifying a ribosomal region was used as an internal yeast standard. The amplification was a 6-plex multiplex reaction, utilizing proprietary primers (ETS Laboratories, St. Helena, CA). Amplification products were run on Beckman CEQ8000 capillary sequencer using standard dye sets and capillary electrophoresis reagents and buffers (Beckman Coulter, Danvers, MA).

A total of 67 isolates originating from grapes, winery equipment and fermentation samples were analyzed. By matching the colony morphologies observed in WL agar with the representative isolates, the population of the most prevalent yeast species (*S. cerevisiae, H. uvarum and Picchia fermentans*) could be estimated.

Analytical methods. The concentrations of glucose, fructose, tartaric acid, malic acid, citric acid and acetic acid were measured by high performance liquid chromatography (HPLC) as described previously (Castellari et al 2000) using an Agilent 1100 series HPLC (Agilent Technologies, Santa Clara, CA, USA). YAN was determined by analyzing AMM and PAN individually, using a Chemwell 2910 Multianalyzer (Unitech Scientific, Hawaiian Gardens, CA) to rapidly test samples. AMM concentration was quantified by using the glutamate dehydrogenase enzymatic test (Ough 1969). Reagents for this test were supplied in the form of enzymatic kits (Unitech Scientific, Ammonia Extended Range UniTAB, 2007). PAN was determined by the o-phthaldehyde/N-acetyl-L-cysteine spectrophotometric assay (NOPA) procedure (Dukes & Butzke 1998) (Unitech Scientific, Primary Amino Nitrogen UniTAB, 2007).

Results

Fermentation Rates and YAN Consumption. The five juices studied had a relatively narrow range of sugar concentrations (17-20 °Brix) and pH (3.00-3.21) (Table 2.1). The range of YAN concentrations, on the other hand, was much wider (68-231 mg N/L).

At Winery 1, initial YAN concentrations ranged from 105 to 144 mg N/L, all at or below the generally accepted minimum for successful fermentation completion (Bisson and Butzke 2000, Bell and Henschke 2005). In fermentation A, a fraction of fermenting juice from the same vineyard was added at day 2, which shortened the lag phase and increased the overall fermentation rate. In fact, this fermentation was complete in 18 days, while the average fermentation time for B and C was 91 days (Figure 2.1). As expected, ammonium ions (AMM) were quickly consumed, and were absent from the fermentation by day 4. Primary amino nitrogen (PAN) consumption followed, although it was never completely exhausted. By the end of fermentation, there was a residual PAN concentration of 11.3 mg N/L, and sugar concentration of 17.9 g/L. Fermentations B and C followed similar patterns: though they did not receive an early addition of fermenting juice, they were blended with new juice from the same vineyards at day 14. Those two fermentations lasted 84 and 98 days, respectively. After consuming 58% of the sugars at day 12, the fermentation rate of B slowed down. Fermentation remained active, though very slow, and was stopped by the winemaker at a residual sugar concentration of 3.6 g/L. By day 9, during the fastest stage of fermentation, AMM was completely consumed. At this point, PAN was also at a minimal concentration (7 mg N/L). It increased with the blending of new juice, then continued to increase until finishing with a concentration of

44.8 mg N/L. Fermentation C also moved quickly until day 5, when 63% of the sugars, and 100% of the AMM, were consumed. Although PAN concentration remained relatively high, fermentation slowed down, finishing with 4.3 g/L of residual sugar at day 98. During this slow period, PAN concentration increased from 62.8 to 75.3 mg N/L.

The two fermentations at Winery 2 showed a marked contrast in YAN. Fermentation D had an initial YAN concentration of 231 md N/L, E had 68 mg N/L, well below recommended ranges for a successful fermentation. In contrast with Winery 1, a long lag phase was observed before fermentation activity started. Fermentation D had a 28-day lag phase, and during this period 25% of the AMM and 29% of PAN were consumed. Once fermentation started vigorously at day 28, the remaining AMM was rapidly taken up, and 63.5 mg/L was consumed in 2 days. PAN was also consumed, but at a slower pace (32 mg N/L in the same time period). At this point, the winemaker decided to make a prophylactic DAP addition equivalent to 100 mg N/L, and by the end of fermentation the final YAN was 165 mg N/L. It was also observed that the two fermentation replicates had different fermentation speeds (Fig 2.2). Tank 2 finished fermentation 13 days after activity started, and tank 1 in 20 days.

Fermentation E had a slightly shorter lag phase of 26 days. Once fermentation started, the rate of sugar consumption was slower than in D. Since the initial AMM concentration was also very low (28.5 mg/L), by the end of lag phase no AMM was left in the medium. At day 28, a 200 mg N/L DAP addition was made, but only 15.3 mg/L of ammonium was consumed after that. PAN concentration increased slightly following the DAP addition, and continued oscillating throughout fermentation, ending up very similar to its initial value (48.3 vs 44.5 mg N/L). Although tank 2 also fermented slightly faster

than tank 1, both fermentations finished at the same time, 50 days after the start of fermentative activity.

Final wine composition indicates that that malolactic fermentation occurred in fermentations B and C (Table 2.3), the two longer fermentations of winery 1. Acetic acid production was consistent across all fermentations, ranging from 0.19-0.27 g/L, except for fermentation D, which produced 0.45 g/L.

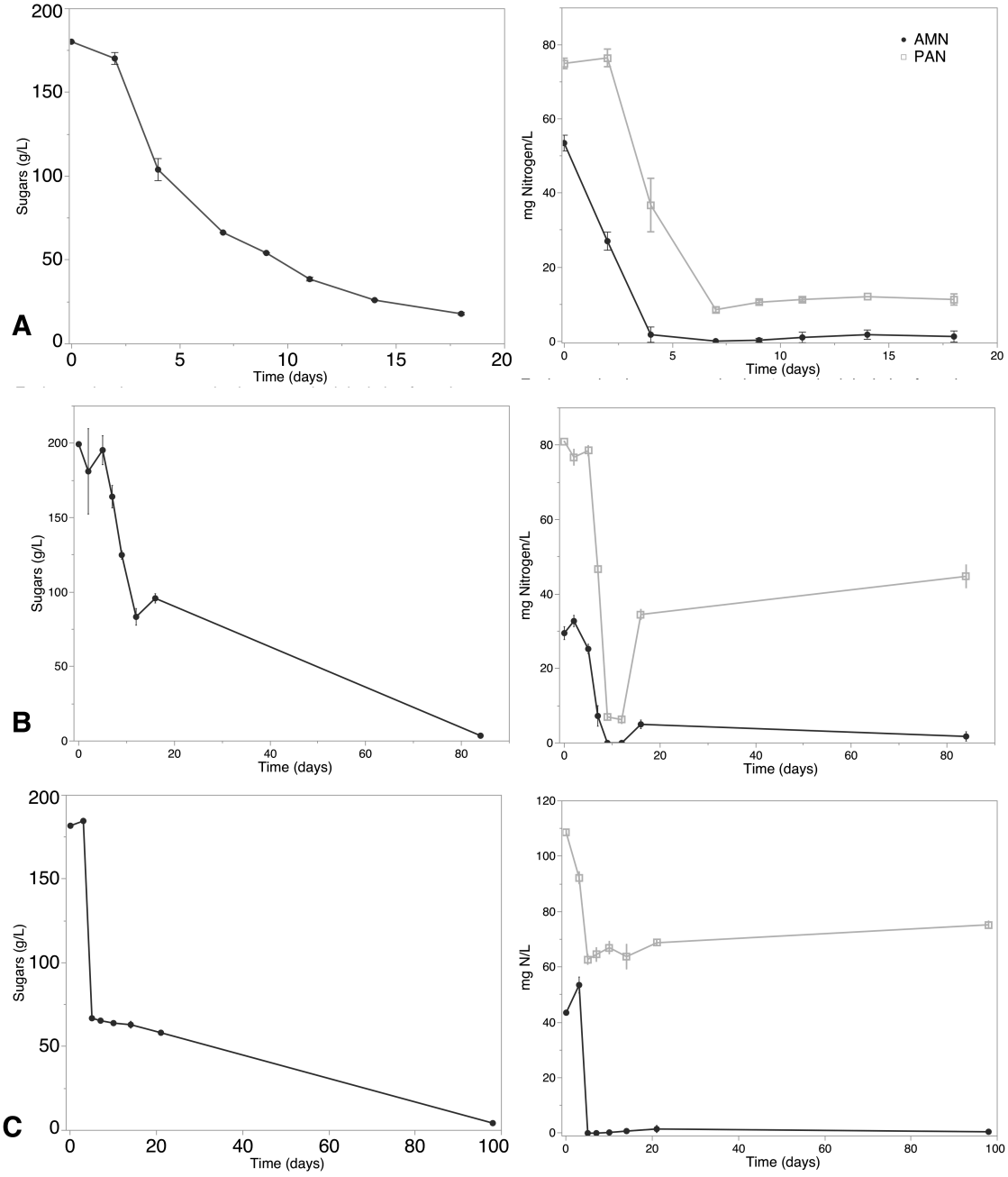

Figure 2.1: Consumption of sugars and YAN components during spontaneous Riesling fermentations A, B and C.

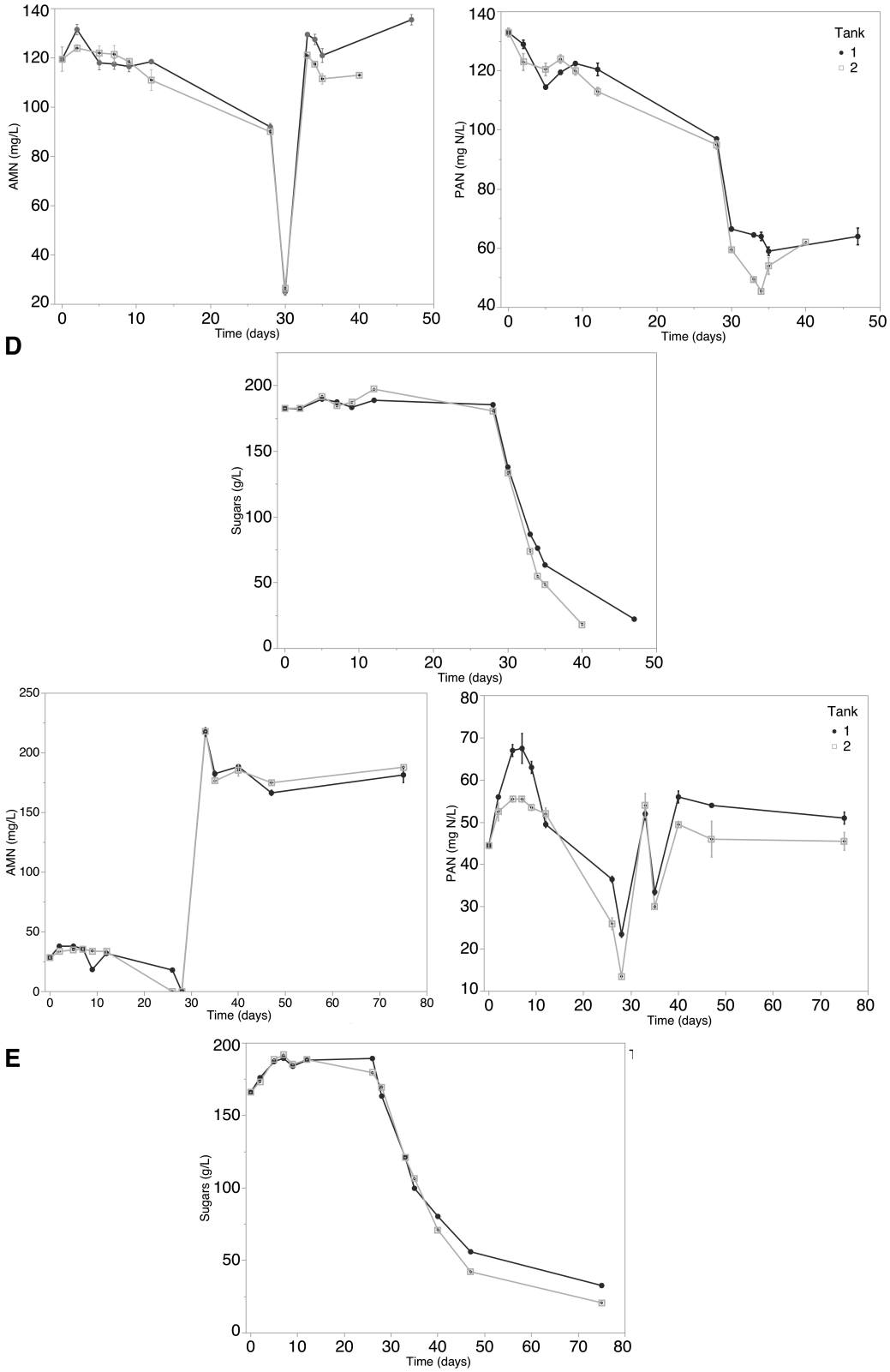

Figure 2.2: Consumption of sugars and YAN components during spontaneous Riesling fermentations D and E.

Table 2.3: Chemical composition of Riesling wines produced by spontaneous fermentation

Parameter	Wines				
	A	B	C	D	E
pH	3.10	3.34	3.18	3.17	3.15
TA[1] (g/L)	9.20	7.76	6.41	10.58	10.99
Ethanol (%v/v)	10.21	11.83	8.72	9.25	9.68
Residual sugar (g/L)	17.9	3.55	4.3	20.3	26.8
Citric acid (g/L)	0.18	0.09	0.06	0.23	0.16
Tartaric acid (g/L)	3.87	2.87	4.36	3.69	4.90
Malic acid (g/L)	3.37	nd	nd	4.26	2.65
Lactic acid (g/L)	nd	3.15	3.09	nd	nd
Acetic acid (g/L)	0.20	0.24	0.27	0.45	0.19

[1]*Expressed at Tartaric Acid Equivalents (TAE)*

Microbial enumeration. Total yeast population found on winery equipment ranged from 10^1-10^4 CFU/mL (Tables 2.4 and 2.5). The tanks at winery 1 had a yeast population ranging from 10^2-10^4 CFU/mL, while in winery 2 no colonies could be recovered.

For three of the five vineyards, the total yeast count number (WL plates) could not be obtained, since the growth of filamentous fungi made colony counting unfeasible. For vineyards A and B, yeast count ranged from 10^2-10^6 CFU/mL.

During fermentation, the viable population of yeast reached maximum values of 10^7-10^8 CFU/mL (Figures 2.3 and 2.4). Most growth curves followed the typical pattern of microorganism growth, characterized by a lag phase, exponential growth, stationary phase, and decline or death phase. Fermentation A, which was blended with a fermenting juice at 2 days, had the most atypical curve. At the first sampling point (beginning of fermentation), total yeast count was already above 10^8 CFU/mL, and fermentation was

past exponential growth phase. After that, the population entered the decline phase, although fermentation of sugars continued. Non-*saccharomyces* yeasts were present in all stages of fermentation, although always in smaller numbers, with a peak of 10^6 CFU/mL. Bacterial populations peaked at 10^5 CFU/mL, followed by a rapid decline.

Table 2.4: Microbial counts for grapes and equipment surfaces at winery 1 determined by direct plating in WL (yeast count) and WL + cycloheximide media (bacteria count)

Sample	Yeast Count (CFU/mL)	Bacteria Count (CFU/mL)
Grapes – vineyard A	5.8E+02	4.0E+05
Grapes – vineyard B	4.8E+06	4.6E+02
Grapes – vineyard C	TNTC[1]	3.6E+04
Sorting table	1.4E+04	4.0E+03
Press	8.4E+02	1.6E+04
Pump	4.0E+03	5.0E+03
Clarification tank	3.0E+02	0
Fermentation tanks	3.1E+04	7.4E+03

[1]*Too numerous to count*

Table 2.5: Microbial counts for grapes and equipment surfaces at winery 2 determined by direct plating in WL (yeast count) and WL + cycloheximide media (bacteria count)

Sample ID	Yeast Count (CFU/mL)	Bacteria Count (CFU/mL)
Grapes – vineyard D	TNTC[1]	3.2E+06
Grapes – vineyard E	TNTC[1]	9.6E+05
Pump	4.0E+01	0
Press	1.7E+04	3.6E+02
Clarification tank	0	0
Fermentation tanks	0	0

[1]*Too numerous to count*

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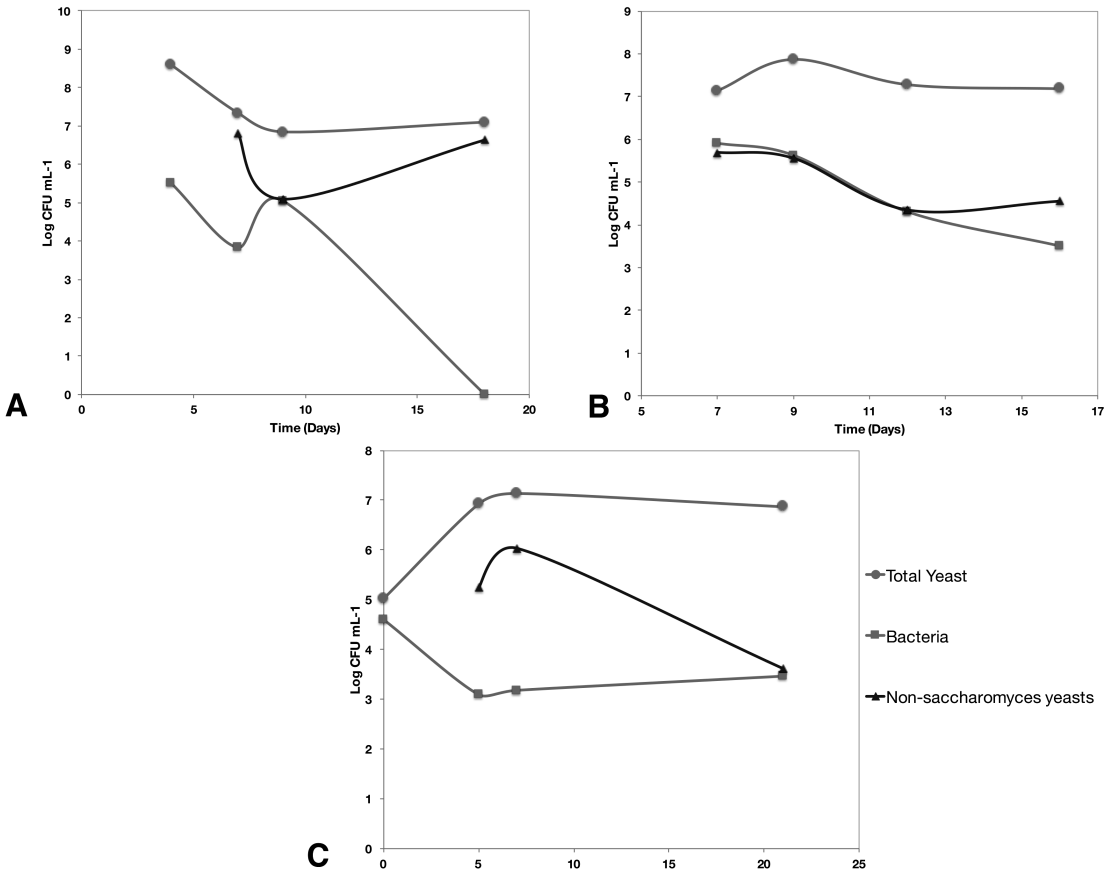

Figure 2.3: Growth of different microbial groups during spontaneous Riesling fermentations at Winery 1.

At fermentations B and C, the exponential growth phase could be observed, reaching 10^7 CFU/mL of total yeast population and then starting to decline. For those two fermentations, it was not possible to take an aseptic sample for microbial analysis of the last day of fermentation (days 84 and 98, respectively), due to scheduling constraints with the commercial winery. However, on the last samples taken, bacterial populations were already in steep decline, totaling 10^3 CFU/mL. Non-*Saccharomyces* populations were also present throughout fermentation, but in a smaller proportion than in fermentation A.

In fermentation B, non-*Saccharomyces* populations ranged from 10^4-10^5 CFU/mL, while in fermentation C, they peaked at 10^6 before rapidly declining to 10^3 CFU/mL.

At Winery 2, the long lag phase can also be observed in the microbial growth curves (Fig. 2.4). The grape juices started with yeast populations in the order of 10^2 CFU/mL. During the exponential phase, a peak population of 10^7-10^8 CFU/mL was reached prior to subsequent decline. Bacterial populations also showed a growth phase in fermentation D, reaching 10^4 CFU/mL before quickly declining. In contrast, fermentation E showed no increase in bacterial count. Non-*Saccharomyces* yeasts were present in all stages of fermentation, reaching 10^8 CFU/mL in fermentation D and 10^7 CFU/mL in fermentation E.

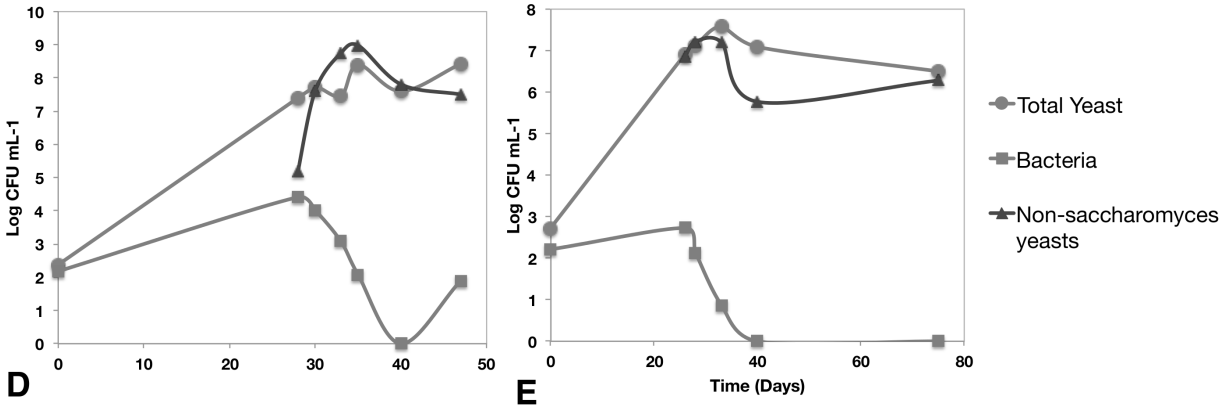

Figure 2.4: Growth of different microbial groups during spontaneous Riesling fermentations at Winery 2.

Microbial isolation and identification. Winery 1 had overall larger yeast populations on equipment surfaces. *H. uvarum* was identified on the isolates from the press, pump and fermentation tanks (Table 2.6). Colony morphology comparison suggested that this species was also present on the other winery equipment sampled

(sorting table and clarification tanks). *S. cerevisiae* was identified in one isolate from a fermentation tank and appeared to be present on all winery surfaces sampled, with populations ranging from 10^2 to 10^4 CFU/mL. In contrast, winery 1 had a non-detectable population of yeast in the clarification and fermentation tanks before use. Two isolates from the press were identified as *Candida* spp. *H. uvarum* was also identified, by colony morphology, on pump and press surfaces at winery 2.

Table 2.6: Distribution of microorganism genus and species isolated from winery equipment – number of isolates

Genus / species	Winery 1 Equipment				Winery 2 Equipment	
	PR	PM	ST	FT	PR	PM
Acetic Acid Bacteria	1			1		
Candida spp.					2	
Hanseniaspora uvarum	1	1		2		
Saccharomyces cerevisiae				1		
Unknown			1			1

PR: press; PM: pump; ST: settling tank; FT: fermentation tank

Initial WL plates with grape samples from vineyards C, D and E developed filamentous fungi. Although it was not possible to obtain a total yeast count for those samples, select isolates were obtained for identification. For the three vineyards in winery 1, the isolates analyzed by VNTR were identified as *Kluyveromyces* spp. and acetic acid bacteria (Table 2.7). By colony morphology, *H. uvarum* was identified in all three vineyards (A, B and C), at 10^4 CFU/mL. In the fermentation samples, *S. cerevisiae* was always the dominant yeast species, reaching 10^8 CFU/mL on fermentation A and 10^7 CFU/mL in fermentations B and C. Nevertheless, non-*Saccharomyces* species were present until late stages of fermentation. In fermentation A, *H. uvarum* was isolated in several fermentation samples until they reached 5°Brix, or approximately 7.7% ethanol.

Kluyveromyces spp was isolated from the grapes and later from the final fermentation sample, which contained 10.2% ethanol. *H. uvarum* was isolated in initial fermentation samples in fermentation B, and was identified by colony morphology in samples until the fermentation reached 5°Brix, or approximately 6.1% ethanol. *Pichia fermentans* was also identified on fermentation B based on colony morphology, surviving until 10°Brix (4.4% ethanol). The same yeast species were isolated from fermentation C: three *H. uvarum* isolates and one unknown *Hanseniaspora* species were identified via VNTR from the clarified juice before fermentation. *H. uvarum* continued to be identified in all fermentation samples based on colony morphology, up to 5°Brix (6.1% ethanol). *P. fermentans* survived until 15°Brix, or 5.6% ethanol.

Table 2.7: Distribution of microorganism genus and species isolated from winery 1 Riesling fermentations – number of isolates

Genus / species	**Vineyard A**					**Vineyard B**		**Vineyard C**			
	G	**BF**	**15B**	**5B**	**EF**	**G**	**BF**	**J**	**BF**	**10B**	**5B**
Acetic Acid Bacteria						1			1	1	1
Hanseniaspora spp.								1			
Hanseniaspora uvarum		1	4	1			1	3			
Kluyveromyces spp.	1				1						
Pichia fermentans										2	
Saccharomyces cerevisiae		1	1	1					1	1	
Unknown	2			3			1	1			1
Saccharomyces spp.					2						

G: grapes; J: clarified juice; BF: beginning of fermentation, 15B: 15°Brix of remaining sugar, 10B: 10°Brix of remaining sugar; 5B: 5°Brix of remaining sugar; EF: end of fermentation

In winery 2, the grape samples from vineyard D contained three isolates from *Kluyveromyces spp*., but this species was not present on vineyard E (Table 2.8). *H.*

uvarum was identified by colony morphology in the grape samples from both vineyards, at populations of 10^6 CFU/mL. Once fermentations started, D had a higher diversity of species and also reached higher population levels than E. The non-*Saccharomyces* species identified in fermentation D by VNTR included *Torulaspora spp*., *H. uvarum*, *Saccharomyces spp*., *Pichia fermentans*, and *Dekkera anomala*. *S. cerevisiae* was the dominant species during fermentation D, reaching a population peak of 10^8 CFU/mL. Of the remaining yeast species present, *H. uvarum* and *P. fermentans* had the most vigorous growth. Both species had a population peak of 10^4 CFU/mL at approximately 2.7% ethanol, and continued to be detected until the fermentation reached 7.2% ethanol, although in declining numbers. For E, *S. cerevisiae* dominated the fermentation, reaching a maximum of 10^7 CFU/mL. The other species present achieved considerably lower numbers. *P. anomala and P. fermentans* were identified by VNTR in the beginning of fermentation, but were not found in later samples. *H. uvarum* was detected by morphology until 3.1% ethanol, but the population did not surpass 10^2 CFU/mL.

Table 2.8: Distribution of microorganism genus and species isolated from winery 2 Riesling fermentations – number of isolates

Genus / species	Vineyard D							Vineyard E	
	G	J	BF	15B	10B	5B	EF	G	BF
Acetic Acid Bacteria								1	
Candida spp.									
Dekkera anomala							1		
Hanseniaspora spp.									
Hanseniaspora uvarum			1			1			
Kluyveromyces spp.	3								
Pichia anomala									1
Pichia fermentans				1	1				1
Saccharomyces cerevisiae			1	1					1
Torulaspora spp		1							
Unknown		1	1			1			1
Saccharomyces spp.			1		1	1			

G: grapes; J: clarified juice; BF: beginning of fermentation, 15B: 15°Brix of remaining sugar, 10B: 10°Brix of remaining sugar; 5B: 5°Brix of remaining sugar; EF: end of fermentation

Strain diversity of *S. cerevisiae*. Fourteen of the isolates identified by VNTR analysis were from the *Saccharomyces* genus: 11 *S. cerevisiae* and 3 from an unknown *Saccharomyces* species (Table 2.9). No single *S. cerevisiae* strain appeared to dominate any of the fermentations; especially in the case of vineyard A, a variety of strains were present at different fermentation stages. Most of the isolates were identified as being closely related to commercial strains. Three strains were isolated in different fermentations from the same winery: Lallemand Enoferm M2, Unknown Strain 2 (both winery 1), and Lalvin Bourgorouge RC212 (winery 2). Additionally, four of the isolates did not match any strain in the ETS database, and were classified as Unknown Strains 1, 2 and 3.

Table 2.9: *S. cerevisiae* strains isolated from winery surfaces and Riesling fermentations

S. cerevisiae strain	Location	Relationship with reference strain[1]
Zymaflore VL1 and Lalvin RWY2	Vineyard A - BF	8/13/5
Unknown strain 1	Vineyard A - 15B	-
Lallemand Enoferm M2	Vineyard A - 5B	5/8/4
Zymaflore F15	Vineyard A- EF	7/9/5
Unknown strain 2	Vineyard A - EF; Fermentation C - 10B	-
QA23/DV1/EC1118	Vineyard B -FT	9/13/7
Lallemand Enoferm M2 and Lalvin W15	Vineyard C - BF	5/8/5
Unknown strain 3	Vineyard D - BF	-
Lalvin Bourgorouge RC212	Vineyard D - 15B	9/14/8
Lalvin Bourgorouge RC212	Vineyard E - BF	9/14/6

BF: beginning of fermentation, 15B: 15°Brix of remaining sugar; 10B: 10°Brix of remaining sugar; 5B: 5°Brix of remaining sugar; EF: end of fermentation; FT: fermentation tank.

[1]Strain alleles/reference strain alleles/ matching alleles.

Discussion

Fermentation Rates and Nitrogen requirements. Commercial fermentations inoculated with *S. cerevisiae* usually take from 6-16 days to reach dryness when initial sugar concentrations are between 200-240 g/L (Boulton et al. 1999, Medina et al. 2013). With the exception of fermentation A, which was finished in 18 days, the other four spontaneous fermentations studied had notably longer fermentation times, ranging from 40 to 98 days. Although the same processing procedures were followed up until crush, distinct fermentation profiles could be observed for the two wineries. This can be attributed to differences in the microbial populations found on grape and winery surfaces, as well as winemaking decisions made during fermentation.

The three fermentations at Winery 1 were characterized by short lag phases, followed by a rapid climb in microbial population and rapid reduction in sugar concentration (Figures 2.1 and 2.3), likely due to larger initial microbial populations

present on winery surfaces and a higher frequency of *S. cerevisiae* isolates found on those fermentations. Because *S. cerevisiae* is the species best adapted to grape juice and the winery environment, and the one with the most efficient fermentative catabolism (Pretorius 2000), culturable levels of this species on winery surfaces likely favors a faster onset of fermentation activity. Fermentation A finished much faster (18 days), likely because it was initially blended with a fermenting juice and started with a higher yeast count. In juices B and C, when approximately 60% of the sugars were consumed, the fermentation rates slowed, likely influenced by low remaining YAN and the natural seasonal cooling of the cellar. Fermentations were concluded in 84 and 98 days (fermentations B and C, respectively).

In contrast, the two fermentations at Winery 2 had a much longer lag phase (27 days on average). Winery 2 had very low yeast counts on equipment surfaces, and no *S. cerevisiae* was isolated from those samples, which might explain why fermentation activity took longer to start. Differences were also observed in fermentation rates between duplicate tanks for the same juice. For each vineyard, juice was settled in a single clarification tank, then racked into two separate fermentation tanks, such that tank 1 received the top part of the clarified juice, and tank 2 the juice and all solids at the bottom. This could explain a faster fermentation rate of tank 2 for both vineyards, either due to a larger yeast population or a higher availability of essential fatty acids on grape solids (Goñi and Azpilicueta 1999).

Clarified juice with moderate sugar concentrations (160-240 g/L), inoculated with commercial *S. cerevisiae* strains, is reported to need between 140 and 260 mg N/L to successfully complete fermentation. (Bely et al. 1990, Cantarelli 1957, Mendes-Ferreira

et al. 2004). In this study, spontaneous fermentations consumed between 59 and 166 mg N/L, generally below the cited minimum limit of 140 mg N/L. A few factors might have contributed for this smaller nitrogen demand. The first is residual sugar concentration; in the studies cited above, RS in successful fermentations was less than 1 g/L, while the wines monitored in this study had on average 8.6 and 23.5 g/L RS (in wineries 1 and 2, respectively). It is a common practice in cool climate winemaking to leave some level of RS to balance the high acidity present in the wines, so fermentations were stopped with total yeast populations ranging from 10^6 to 10^8 CFU/mL. If the fermentations had been allowed to continue and had reached lower sugar levels, it is likely that the final YAN concentration would be lower. Additionally, the microbiome of spontaneous fermentations consists of various yeast species and strains. It has been shown that wild species tend to have lower nitrogen requirements than commercial *S. cerevisiae* strains, though they also present slower fermentation rates (Medina et al. 2012, Barrajon-Simancas et al. 2011). Lastly, towards the middle and late stages of fermentation, most of the species that initiate fermentation enter death-phase, and subsequently may release amino acids back into the fermentation medium (Alexandre et al. 2001).

The long fermentation times observed in this study are likely attributed to the absence of an initial inoculum, yeast antagonistic interactions, and competition for nutrients. Studies analyzing the effect of inoculating mixed cultures of *S. cerevisiae* and non-*Saccharomyces* yeasts have found that the fermentation time increased in the mixed cultures, and that addition of nitrogen and a vitamin mixture helped to increase fermentation rates (Medina et al. 2012, Barrajón-Simancas et al. 2011). In this study, the fact that DAP added to fermentations D and E was not consumed suggests that

competition for nitrogen was likely not a factor, although it is possible that the added nitrogen was not utilized because fermentations were past the growth phase. It is also possible that exhaustion of one or more micronutrients contributed to the long fermentation times. The final acetic acid concentration of wine D was notably higher than the other wines, which could have been caused by excessive DAP addition.

Microbial Characterization. The differences in fermentation profiles between wineries can be explained by differences in microbial ecology. Winery 1 had higher populations of resident microflora, including *S. cerevisiae*, and lower populations of non-*Saccharomyces* yeasts during fermentation. The low populations of resident micro flora on equipment in Winery 2 (Table 2.6) likely resulted in higher participation of grape-derived microorganisms in fermentation. The species isolated from Winery 2 equipment were *Candida* spp., which could be autochthonous from grape surfaces or from human contact, and *H. uvarum*, which is grape-derived. *S. cerevisiae* is known to be present in very low numbers on the surface of healthy grapes, and can not be detected by direct plating (Mercado et al. 2007). Even after fermentation started, a higher diversity of *non-Saccharomyces* yeasts was isolated from those fermentations, suggesting that wine microflora was predominately vineyard-derived. Nevertheless, when comparing the two fermentations at Winery 2, we can see that fermentation D reached a higher population peak, and finished in less time than fermentation E. This could be explained by the very low initial YAN of juice E (68 mg N/L).

Strain diversity of *S. cerevisiae*. Winery 1 showed a higher diversity of yeast strains, with 6 different strains identified across fermentations (Table 2.9). Only one of these strains, reportedly similar to EC1118, was isolated from equipment, and the

remaining from fermenting juice. Though some studies have recovered *S. cerevisiae* from grape samples utilizing enrichment methods, the number found on fruit is very small, and the few strains found on grapes are rarely isolated from corresponding fermentations (Mercado et al. 2007). Hence, it is likely that the active yeasts identified in the wines were part of the resident microflora of Winery 1. There appeared to be no single dominant strain throughout fermentations, a finding consistent with previous research reporting a high diversity of *S. cerevisiae* strains and no clear dominance (Mercado et al. 2007, Csoma et al. 2010, Torija et al. 2001). However, due to the small number of isolates analyzed, relative abundances of each strain at the different stages of fermentation could not be determined. Only two strains were isolated from Winery 2: one closely related to Lalvin Bourgorouge RC212 (Lallemand), and another that did not match any commercial strain on ETS's database. No *S. cerevisiae* was isolated from Winery 2 grapes or equipment surfaces, making it hard to infer the origin of the *S. cerevisiae* isolated from fermentation samples. Although recent studies have shown that *S. cerevisiae* populations from vineyard and winery environments can mix freely, winery resident populations tend to have a greater contribution to fermentation, since they are present in greater numbers and are more adapted to the fermentation environment (Bisson 2012).

A total of 4 isolates, with 3 different patterns, could not be matched to any commercial strain on ETS's database. These could be native *S. cerevisiae* strains, although a more complete DNA sequencing must be done in order to confirm this hypothesis. The remaining seven isolates were closely related to commercial *S. cerevisiae* strains, some of which have been or are in use in the wineries for inoculated

fermentations (e.g. EC1118, W15 and RC212). Those isolates might have originated from commercial strains retained on winery surfaces that gradually underwent genotypic changes through such mechanisms as spontaneous mutation, genetic drift, or environmental selection (Bisson 2012).

Conclusion

The five spontaneous fermentations studied consumed less YAN than the average reported for inoculated fermentations. Slower fermentation rates, as well as higher levels of residual sugars, are likely determinants for this smaller consumption, but the contribution of factors such as nitrogen requirements of different non-*Saccharomyces* yeasts, and nitrogen release during death phase of those populations, needs to be further investigated.

The microflora characterized from equipment surfaces were substantially different in the two wineries studied. Winery 1 equipment had higher yeast counts and a greater diversity of species, including *S. cerevisiae*. The shorter lag phase and the high sugar consumption rates in the beginning of fermentation also suggest a stronger dominance of *S. cerevisiae*, most likely originating from the winery. Winery 2 had low microbial populations on equipment surfaces, long lag phases and a higher diversity of non-*Saccharomyces* yeasts during fermentation. This suggests a larger influence of grape-derived microflora in Winery 2. Further research is needed to confirm the origin of fermentation microflora on both wineries. Although *S. cerevisiae* dominated all fermentations, non-*Saccharomyces* yeasts were isolated even in late stages, showing tolerance to ethanol concentrations of 6-7%. The majority of *S. cerevisiae* isolates

obtained were similar to commercial strains, some of which had been used before by the wineries. The diversity of strains isolated from different stages of fermentation suggests that there was no single strain dominating fermentation, but that several strains were involved.

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CHAPTER 3
EXPERIMENTAL MODIFICATIONS AND FUTURE WORK

The influence of nitrogen compounds in fermentation performance and wine composition is widely acknowledged, nevertheless few studies have focused in investigating YAN requirements of cool-climate fermentations. Additionally, there is growing interest in conducting spontaneous fermentations in the Finger Lakes region, but little is known about microbiome composition and nutrient requirements of such fermentations. This research provided YAN recommendations better suited for cool-climate winemaking, based on commonly used yeast strains, and presented new information on the microbial ecology of spontaneous fermentations in the NY Finger Lakes region, as well as their nitrogen requirements.

YAN Optimization for cool-climate Riesling

Considering the limited benefits that were observed with ammonium nitrogen (DAP) supplementation, future research should focus on investigating possible benefits of supplementation with other nitrogen sources, namely primary amino acids. Given that amino acid metabolism is directly involved in the synthesis of higher alcohols and their corresponding ethyl and acetate esters via the Ehrlich pathway, addition of different amino acid mixtures can potentially modulate production of different volatiles. Further, since ammonium ions are more easily taken up by yeasts, addition of ammonium to the juice tends to increase nitrogen utilization, but may delay and reduce utilization of amino acids. Therefore, the ratio of organic to inorganic nitrogen of the juice also has an impact on nitrogen metabolism by yeast. Direct additions of amino acids to grape must are not allowed in the US; however, commercial

preparations including both inorganic nitrogen and amino acids are available. More research is needed to investigate the effect of those preparations in wine volatile composition.

To improve the design of this experiment, a full quantification of wine volatiles could be conducted instead of the semi-quantification performed here. By obtaining the absolute concentrations of volatiles instead of relative concentrations, it would be possible to compare the results with published sensory thresholds and postulate which compounds were likely to have a larger impact on wine aroma. The sensory evaluation could also include descriptive analysis, where the intensity of select aroma attributes would be rated by trained panelists. This could also provide more information regarding which aromas were being affected by YAN supplementation. Lastly, metabolomic studies could be conducted in the fermentation and wine samples, to better understand the relationship between amino acid composition of grape juice, their consumption and synthesis of volatile fermentation products.

Spontaneous Fermentations:

This study confirmed that the spontaneous fermentations analyzed had a diverse microbial ecology, with different *S. cerevisiae* strains and participation of non-*Saccharomyces* yeasts until late stages of fermentation. However, it was not possible to determine the origin of fermentation microflora in each winery. The same wineries will continue to be monitored over the next two harvests to augment the findings presented above. By comparing data from consecutive years, it may be possible to confirm whether certain strains are part of the resident winery microflora, and if there are changes associated with vintage characteristics such as weather patterns.

To avoid the issue with mold contamination in the grape samples, the vineyard sampling procedure should move to berry samples instead of whole clusters. With that method, any bunch rot present in the clusters can be sorted out of the samples. Additionally, agents to inhibit filamentous fungi, such as rose bengal or chloramphenicol, should be added to the growth media to prevent mold contamination. The frequency of sampling could also be modified in fermentations with long lag phases, such as D and E. Although there was no decrease in sugar concentration for the first 30 days, reduction in YAN content indicates that microbial populations were metabolically active during this period. Therefore, sampling for microbial characterization during lag phase could provide important information regarding the populations present in early fermentation stages.

In the following years, a higher number of isolates will be analyzed, so that microbial counts of each genus present throughout fermentation can be obtained, as well as a relative proportion of each *S. cerevisiae* strain present. Additionally, nitrogen consumption patterns can be evaluated, to assess whether they are consistently below the consumption values of inoculated fermentations. A more complete genotype sequencing of the unknown *S. cerevisiae* strains that were isolated will help confirm whether they are in fact indigenous strains, and investigate possible relationships between the microbial community executing fermentations to the sensory properties of final wines.