The impact of grape clone, yeast strain and protein on sparkling wine quality

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Abstract

The foaming properties in sparkling wine are an indicator of quality as it is the first quality perception consumers have upon opening a bottle of sparkling wine. Proteins, which are derived from both grapes and yeast during sparkling wine production, are known to impact the foaming properties in finished sparkling wines. The objectives of this project were to (1) understand the role and relationship that proteins have on the overall foaming properties and overall quality in sparkling wine, (2) determine the role different yeast and grape clones from different varietals have on sparkling wine quality and (3) understand how bentonite affects sparkling wine quality. The protein concentration in sparkling wine produced from Mariafeld Pinot noir appeared to impact the foaming properties. The longest elapsed time for foam dissipation was observed in the control treatment where bentonite was not used to strip protein and the shortest time was observed in the treatment where bentonite was used to remove grape and yeast proteins. In Riesling sparkling wines, the largest protein concentrations were observed in non-bentonite treated juices while the lowest were observed in the bentonite treatments. The prevalence of foam observed in both bentonite treatments, where grape proteins were completely removed, indicated that proteins derived over the course of secondary fermentation were foam forming in Riesling sparkling wine. It was also observed that different yeast, varietals, clones and soil compositions may impact the protein concentrations, chemical compositions and overall quality of sparkling wine. The results of this research aim to better understand sparkling wine quality to optimize production in the Niagara Peninsula.
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# Table of Contents

## Chapter 1

### 1.1. Introduction and Literature Review

- Overview of sparkling wine production .......................................................... 1
- Varietals used in sparkling wine production .................................................... 3
- The Secondary Fermentation ............................................................................ 4
- Lees aging ........................................................................................................ 5
- Sparkling wine production in cool climate regions .......................................... 6
- Factors affecting sparkling wine quality ......................................................... 8
- Vineyard conditions on sparkling wine quality ............................................. 8
- Aging on sparkling wine quality ................................................................... 8
- Oenological practices on sparkling wine quality .......................................... 9
- Fermentation by-products on sparkling wine quality ...................................... 10

### 1.2. Assessing sparkling wine quality ............................................................... 11

- Foam parameters ............................................................................................ 11
- Foaming assessments ....................................................................................... 12

### 1.3. Role of proteins, grape varietal and yeast on sparkling wine quality ....... 17

- Role of proteins in sparkling wine ................................................................. 17
- Role of grape varietal and clones in sparkling wine ....................................... 20
- Role of yeast in sparkling wine ...................................................................... 21

### 1.4. Summary .................................................................................................. 24

### 1.5. Thesis objectives and hypotheses ............................................................. 26

### 1.6. Literature Cited ....................................................................................... 27

## Chapter 2

2. The role of grape and yeast proteins on sparkling wine quality produced from Mariafeld Pinot noir and two yeast species .................................................................... 32

- Abstract ............................................................................................................ 32
- Introduction ...................................................................................................... 34
- Materials and Methods .................................................................................. 36
  - Experimental Design .................................................................................... 36
2.3.2. Yeast strains........................................................................................................37
2.3.3. Grape juice..........................................................................................................37
2.3.4. Chemical analyses of juice and wine..................................................................37
2.3.5. Protein analyses of juice and wine......................................................................38
2.3.6. Sample collection and timepoints........................................................................40
2.3.7. Primary fermentation and winemaking...............................................................40
2.3.8. Yeast inoculation procedure for tirage and secondary fermentations..............41
2.3.9. Foaming analysis .................................................................................................44
2.3.10. Statistical analysis..............................................................................................44
2.4. Results ....................................................................................................................45
  2.4.1. Chemical analysis of initial juice and base wine...............................................45
  2.4.2. Liqueur de tirage buildup....................................................................................46
  2.4.3. Chemical analysis of final sparkling wines.......................................................48
  2.4.4. Protein analysis of juice and wine.......................................................................49
  2.4.5. Foaming analysis of final sparkling wines.........................................................59
2.5. Discussion ................................................................................................................61
  2.5.1. Challenges in culture buildup for secondary fermentation.............................61
  2.5.2. Protein profile and concentration during sparkling wine production................62
  2.5.3. Foaming properties in finished sparkling wine..................................................63
2.6. Conclusion ...............................................................................................................64
2.7. Literature Cited .......................................................................................................66

Chapter 3 ......................................................................................................................68
3. The role of grape and yeast proteins on Riesling sparkling wine quality..................68
3.1. Abstract ...................................................................................................................68
3.2. Introduction .............................................................................................................70
3.3. Materials and Methods ..........................................................................................72
  3.3.1. Experimental Design. .......................................................................................72
  3.3.2. Yeast ..................................................................................................................73
  3.3.3. Grape juice ........................................................................................................73
  3.3.4. Chemical analyses of juice and wine...............................................................73
  3.3.5. Protein analyses of juice and wine....................................................................74
3.3.6. Sample collection and timepoints ................................................................. 76
3.3.7. Primary fermentation and winemaking .......................................................... 76
3.3.8. Yeast inoculation procedure for tirage and secondary fermentations ............. 77
3.3.9. Foaming analysis ......................................................................................... 80
3.3.10. Statistical analysis ..................................................................................... 80
3.4. Results ............................................................................................................. 80
3.4.1. Chemical analysis of initial juice and base wine ........................................... 80
3.4.2. Liqueur de tirage .......................................................................................... 82
3.4.3. Chemical analysis of final sparkling wines .................................................... 83
3.4.4. Protein analysis of juice and wine ............................................................... 84
3.4.5. Foaming analysis of final sparkling wines ................................................... 94
3.5. Discussion ......................................................................................................... 96
3.5.1. Challenges in culture buildup for secondary fermentation ............................ 96
3.5.2. Protein profile and concentration during sparkling wine production ............ 98
3.5.3. Foaming properties in finished sparkling wine ............................................. 100
3.6. Conclusion ....................................................................................................... 102
3.7. Literature Cited ............................................................................................... 103

Chapter 4 ............................................................................................................... 105
4. The role of grape varieties and grape clones on sparkling wine quality .................. 105
4.1. Abstract .......................................................................................................... 105
4.2. Introduction ..................................................................................................... 107
4.3. Materials and Methods ................................................................................... 109
4.3.1. Experimental Design .................................................................................. 109
4.3.2. Yeast ......................................................................................................... 110
4.3.3. Grape juice ............................................................................................... 110
4.3.4. Chemical analyses of juice and wine ......................................................... 111
4.3.5. Protein analyses of juice and wine ............................................................ 111
4.3.6. Sample collection and timepoints ............................................................ 113
4.3.7. Primary fermentation and winemaking ...................................................... 113
4.3.9. Statistical analysis ...................................................................................... 116
4.4. Results ............................................................................................................. 117
4.4.1. Chardonnay: Chemical analyses, fermentation kinetics and protein analyses. ........ 117
4.4.2. Chardonnay musqué: Chemical analyses, fermentation kinetics and protein analyses.
....................................................................................................................................... 123
4.4.3. Pinot noir: Chemical analyses, fermentation kinetics and protein analyses. .......... 128
4.4.4. Riesling: Chemical analyses, fermentation kinetics and protein analyses. .......... 132
4.5. Discussion .................................................................................................................. 137
  4.5.1. Chardonnay........................................................................................................ 137
  4.5.2. Chardonnay musqué ......................................................................................... 139
  4.5.3. Pinot noir ....................................................................................................... 140
  4.5.4. Riesling ......................................................................................................... 141
  4.5.5. Varietal comparison and potential foaming properties .................................. 143
4.6. Conclusion ................................................................................................................ 144
4.7. Literature Cited ...................................................................................................... 145

Chapter 5 ....................................................................................................................... 148
5. Discussion and Conclusions .................................................................................... 148
  5.1 Literature Cited ..................................................................................................... 155

Appendix I: Protein Normalization and Quantification from SDS-PAGE gels ............ 157
Appendix II: Additional results from Chapter 2 ....................................................... 158
Appendix III: Additional results from Chapter 3 ..................................................... 159
List of Tables

Chapter 2

Table 2.1: Chemical composition of Mariafeld Pinot noir prior to fermentation and after primary fermentation of juice to base wine.......................................................... 45

Table 2.2: Cell viability (%) and specific gravity of S. cerevisiae during Stages 2 and 3 of liqueur de tirage culture buildup for secondary fermentation in bottle................................................. 46

Table 2.3: Cell viability (%) of S. bayanus during Stages 2 and 3 of tirage culture buildup for secondary fermentation in bottle.................................................. 47

Table 2.4: Chemical composition of Mariafeld Pinot noir wine treatments........................................ 48

Table 2.5: Protein concentration in finished sparkling wines (S. cerevisiae treatments) determined from BCA assay and densitometry from SDS-PAGE........................................... 59

Chapter 3

Table 3.1: Chemical composition of Riesling juice after treatment and prior to and after primary fermentation into the base wine.......................................................... 81

Table 3.2: Cell viability (%) and specific gravity of S. cerevisiae during Stages 2 and 3 of tirage culture buildup for secondary fermentation in bottle................................................. 82

Table 3.3: Chemical composition of finished Riesling treatments................................................ 83

Table 3.4: Protein concentration in finished sparkling Riesling wines determined from BCA assay and densitometry from SDS-PAGE.................................................. 93

Chapter 4

Table 4.1: Chemical composition of Chardonnay clonal treatments on pre-inoculated juice, post-ferment base wine and at the lees aging midpoint .............................................. 118

Table 4.2: Protein concentration in Chardonnay clones at lees aging midpoint determined from BCA assay and densitometry from SDS-PAGE.................................................. 122

Table 4.3: Chemical composition of Chardonnay musqué soil treatments on pre-inoculated juice, post-ferment base wine and at the lees aging midpoint.............................................. 123

Table 4.4: Protein concentration in Chardonnay musqué soil treatments at lees aging midpoint determined from BCA assay and densitometry from SDS-PAGE.............................................. 127

Table 4.5: Chemical composition of Pinot noir clone treatments on pre-inoculated juice, post-fermentation base wine and at the lees aging midpoint.............................................. 128

Table 4.6: Protein concentration in Pinot noir clonal treatments at lees aging midpoint determined from BCA assay and densitometry from SDS-PAGE.............................................. 132

Table 4.7: Chemical composition of Riesling treatments on pre-inoculated juice, post-fermentation base wine and at the lees aging midpoint.............................................. 133
Table 4.8: Protein concentration in Riesling treatments at lees aging midpoint determined from BCA assay and densitometry from SDS-PAGE.
List of Figures

Chapter 1

Figure 1.1: Production steps required in traditional sparkling winemaking ........................................... 3
Figure 1.2: Setup of CAVE system used to measure the foaming properties in sparkling wines. 13
Figure 1.3: Schematic of the equipment to measure the foaming properties in wine using the Mosalux® method. ......................................................................................................................................................... 14
Figure 1.4: Depiction of the Rudin tube setup ........................................................................................... 15
Figure 1.5: FIZZeyeRobot schematic to determine foam parameters. ...................................................... 16
Figure 1.6: Foam structure and distribution of glycoproteins ................................................................ 18

Chapter 2

Figure 2.1: Experimental design and breakdown of treatments of sparkling wine produced from Mariafeld Pinot noir .................................................................................................................................................. 36
Figure 2.2: Protein profile over the course of primary fermentation of Mariafeld Pinot noir juice to base wine ....................................................................................................................................................... 49
Figure 2.3: Total protein concentration in pre-inoculated Mariafeld Pinot noir juice and post-fermented base wine determined using densitometry from SDS-PAGE gels ........................................ 50
Figure 2.4: Protein profile of control/no treatment and bentonite treated at a given time point in the sparkling wine production of Mariafeld Pinot noir .................................................................................. 51
Figure 2.5: Total protein concentration of control/no treatment and bentonite treatment over four stages in primary fermentation determined using densitometry from SDS-PAGE gels ...... 52
Figure 2.6: Protein profile over the course of three timepoints in secondary fermentation of Mariafeld Pinot noir for all control base wine treatments. ......................................................... 53
Figure 2.7: Protein profile over the course of three timepoints in secondary fermentation of Mariafeld Pinot noir for all bentonite base wine treatments ........................................................................... 54
Figure 2.8: Total protein profile concentration over the course of secondary fermentation in bottle of Mariafeld Pinot noir base wine to finished sparkling wine (A) inoculated with S. cerevisiae EC 1118 (B) inoculated with S. bayanus Brock isolate and determined using densitometry from SDS-PAGE gels ......................................................................................................................................................... 55
Figure 2.9: Protein profile of final Mariafeld Pinot noir wine comparing the addition of Inoclair 2/bentonite in tirage ......................................................................................................................................................... 57
Figure 2.10: Total protein profile concentration of final Mariafeld Pinot noir wine treatments determined using densitometry from SDS-PAGE gels .......................................................................................................................... 58
Figure 2.11: Protein profile of all Mariafeld Pinot noir sparkling wine treatments ................................ 59
Figure 2.12: Foaming analysis of finished Pinot noir Mariafeld sparkling wine treatments.............. 60
Chapter 3

Figure 3.1: Experimental design and breakdown of treatments of sparkling wine produced from Riesling grapes................................................................. 72
Figure 3.2: Protein profile over the stages of base wine production: initial pressed Riesling juice to after settling, racked and pre-inoculated juice and the profile over fermentation of the pre-inoculated juice to post-fermentation base wine............................................. 85
Figure 3.3: (A) Protein concentration in initial pressed Riesling juice treatments and post-treatment and pre-inoculated juice. (B) Protein concentration in before and after primary fermentation of Riesling treatments determined using densitometry from SDS-PAGE gels................................. 86
Figure 3.4: Protein profile of control/no treatment and bentonite treated at a given time point in Riesling sparkling wine production .................................................................................. 87
Figure 3.5: Protein concentration of Riesling treatments at a given time point before and after primary fermentation determined using densitometry from SDS-PAGE gels................................. 88
Figure 3.6: Protein profile over the course of three timepoints in secondary fermentation for Riesling .................................................................................................................. 89
Figure 3.7: Protein concentration over the course of secondary fermentation in bottle of Riesling base wine to finished sparkling wine determined using densitometry from SDS-PAGE gels. .... 90
Figure 3.8: Protein profile of final Riesling sparkling wine comparing the addition of Inoclar 2 in tirage ........................................................................................................................................... 91
Figure 3.9: Protein profile concentration of final Riesling wine treatments determined using densitometry from SDS-PAGE gels. .............................................................. 92
Figure 3.10: Protein profile of all Riesling sparkling wine treatments ................................................................................................................................................................................... 94
Figure 3.11: Foaming analysis of finished Riesling sparkling wine treatments.................. 95

Chapter 4

Figure 4.1: Breakdown of grape varietals and clones used in sparkling clonal trial. ............ 109
Figure 4.2: Primary fermentation kinetics of all Chardonnay juice clones inoculated with S. cerevisiae EC 1118. ......................................................................................................................................... 119
Figure 4.3: Protein profile of Chardonnay clones (95, 548, 127 and 128) at three winemaking timepoints.......................................................................................................................... 120
Figure 4.4: Protein profile concentration over the course of sparkling wine production of Chardonnay clonal treatments and determined using densitometry from SDS-PAGE gels. ..... 121
Figure 4.5: Primary fermentation kinetics of all Chardonnay musqué juice treatments inoculated with S. cerevisiae EC 1118.. .................................................................................................. 124
Figure 4.6: Protein profile of Chardonnay musqué (clone 809) soil treatments at three winemaking timepoints......................................................................................................................... 125
Figure 4.7: Protein concentration over the course of sparkling wine production of Chardonnay musqué soil treatments and determined using densitometry from SDS-PAGE gels. ............ 126
Figure 4.8: Primary fermentation kinetics of all Pinot noir juice clones inoculated with *S. cerevisiae* EC 1118. ................................................................. 129

Figure 4.9: Protein profile of Pinot noir clones at three winemaking timepoints. .................. 130

Figure 4.10: Protein concentration over the course of sparkling wine production of Pinot noir clone treatments and determined using densitometry from SDS-PAGE gels. ................................. 131

Figure 4.11: Primary fermentation kinetics of all Riesling juice treatments inoculated with *S. cerevisiae* EC 1118. ................................................................. 134

Figure 4.12: Protein profile of Riesling clones and soil treatments at three winemaking timepoints ................................................................................................................... 135

Figure 4.13: Protein concentration over the course of sparkling wine production of Riesling treatments and determined using densitometry from SDS-PAGE gels. ............................... 136
Chapter 1

1.1. Introduction and Literature Review

1.1.1. Overview of sparkling wine production

Sparkling wine production spans over thirty countries worldwide and represents approximately 7% of the total wine production in the world (Robillard and Marchal 2015). Sparkling wine is differentiated from still wine by the foam or effervescence that is formed during the pouring of the wine into a glass (Cilindre et al. 2010). Effervescence is defined as the steady release of dissolved carbon dioxide gas molecules that are responsible for bubble formation (Pozo-Bayón et al. 2009). Due to this effervescence, sparkling wine is not considered in the same category as still or table wines. Sparkling wine is classified under special wines, the same category as fortified wines which include port and sherry (Robillard and Marchal 2015). The origin of foam found in sparkling wines depends on the production method. Production costs, quality and cost of wine are dependant of the production method. The four main ways to produce sparkling wine include: the traditional method, Charmat method, transfer method and the carbonation method (Kemp et al. 2017). Some examples of sparkling wines produced in the traditional method are Champagne, the origin of sparkling wine, and Crémant from France as well as Cava from Spain (Iland et al. 2009). The traditional method consists of two stages (Cilindre et al. 2010; Guillamón et al. 2015; Martí-Raga et al. 2015). The first fermentation or primary fermentation consists of inoculating the grape juice with a yeast starter culture which ferments into a base wine. The second fermentation occurs when the base wine is inoculated with a liqueur de tirage, yeast culture, and fermented in bottle (Cilindre et al. 2010; Guillamón et al. 2015; Martí-Raga et al. 2015). Traditional sparkling wines typically undergo secondary fermentation with a strain of S. cerevisiae yeast (Ribéreau-Gayon et al. 2006). Secondary fermentation is where the sparkling or bubbles are produced as yeast consume sugar and their by-products ethanol and carbon dioxide are released into the wine: $\text{C}_6\text{H}_{12}\text{O}_6 \rightarrow$
2\text{CH}_3\text{CH}_2\text{OH} + 2\text{CO}_2 \text{ (Ribéreau-Gayon et al. 2006).} \text{ The resulting sparkling wine typically has a pressure ranging from 5-7 atm. The Charmat method involves primary fermentation of juice into base wine and secondary fermentation of the base wine in a tank that is resistant to pressure. The resulting pressurized sparkling wine, around 2-4 atm, is filtered and then bottled. The transfer method is a combination of both traditional and Charmat procedures. After the base wine is made, a } \textit{liqueur de tirage} \text{ mixture is added and secondary fermentation occurs in bottle. Once secondary fermentation and lees aging has completed, the bottles are opened and wine is put into a pressurized tank. The resulting sparkling wine is then rebottled. The carbonation method has the lowest production costs as it involves only one fermentation followed by carbonating still wine in a tank (Stevenson 2005). } \textbf{Figure 1.1.} \text{ depicts the production flow and processing steps required for traditional sparkling winemaking.}
1.1.2. **Varietals used in sparkling wine production**

Traditional varietals that are used in Champagne or in traditional method production are Chardonnay, Pinot noir and Pinot meunier. Wine regions that do not grow large quantities of these varietals or are not able to successfully grow these varietals for sparkling wine production may resort to using other varietals of the *Vitis vinifera* origin such as Riesling, Shiraz, Merlot, and Pinot gris. Once grapes are harvested and primary fermentation is complete, a desirable base wine should have approximately 10-11% (v/v) alcohol concentration (Stevenson 2005; Borrull *et al.* 2015). Base wines higher than 11% (v/v) alcohol may cause problems for the *liqueur de tirage* culture in
secondary fermentation. Furthermore, a pH ranging from 3.0 to 3.3 and a titratable acidity between 10-12 g L\(^{-1}\) tartaric acid are ideal parameters in sparkling base wine (Ribéreau-Gayon \textit{et al.} 2006).

1.1.3. The Secondary Fermentation

The steps involved to start secondary fermentation in bottle with the \textit{liqueur de tirage} include: adding sugar (typically sucrose at around 20-25 g L\(^{-1}\)), an adjuvant (typically bentonite) to promote removing of yeast cells after aging and flocculation, and a yeast starter culture (Guillamón \textit{et al.} 2015; Martí-Raga \textit{et al.} 2015). To overcome these stresses and successfully undergo secondary fermentation, the yeast population must be appropriately acclimated prior to being introduced to the base wine and bottled (Martí-Raga \textit{et al.} 2015). The yeast population go through an adaption process known as \textit{pied-de-cuve} where the selected yeast is grown and introduced to several media containing increasing concentrations of ethanol (Laurent and Valade 2007). There is no standard recipe for sugar, nutrients or ethanol medium set for the \textit{pied-de-cuve}. The \textit{pied-de-cuve} is a two-stage process consisting of (1) adaptation and (2) proliferation phases (Martí-Raga \textit{et al.} 2015). In the adaptation phase, yeast is introduced to smaller concentrations of ethanol along with nutrients and sugar additions and in proliferation introduced to higher amounts of ethanol along with nutrients and sugar to be acclimated to the concentration of ethanol in the base wine for secondary fermentation. The yeast strain used and the build up procedure for the \textit{pied-de-cuve} directly affects kinetics in secondary fermentation (Martí-Raga \textit{et al.} 2015).

One main issue in sparkling wine production is the stresses yeast endure during secondary fermentation in bottle. Some of these stresses yeast tolerate include ethanol, temperature, sulfur dioxide, low oxygen availability and increasing CO\(_2\) concentration (Guillamón \textit{et al.} 2015; Martí-Raga \textit{et al.} 2015; Borrull \textit{et al.} 2016). Not much research has been done to understand the kinetics and developments of secondary fermentation and how it may be influenced by factors affecting
the first fermentation (Guillamón et al. 2015). The temperature and yeast strain used for still wine fermentation have been shown to impact fermentation development and final properties in wine (Martí-Raga et al. 2015). The nitrogen content has been shown to not only affect the development of the first fermentation, but the organoleptic properties in finished sparkling wine as well as yeast viability (Bell and Henschke 2005). The CO₂ pressure has been proven to strongly inhibit biomass production and the fermentative activity in yeast (Martí-Raga et al. 2015). This inhibition can only be reduced when yeast is appropriately adapted to wine prior to the start of secondary fermentation in bottle (Martí-Raga et al. 2015).

1.1.4. Lees aging
The higher quality and priced traditional method sparkling wines, including Champagne, are known for having longer aging on lees (Rowe et al. 2010). Lees is defined as the residue that forms at the bottom or sides of the wine bottle after secondary fermentation and during aging (Riu-Aumatell et al. 2015). Lees consists of microorganisms (predominately yeast), phenolic compounds, inorganic matter and tartaric acid (Riu-Aumatell et al. 2015). It is common for wines produced in the traditional method to have lees kept in bottle for years after the completion of secondary fermentation. A minimum of 15 months of lees aging is required for Champagne and 9 months of lees aging for Cava (Vincenzi et al. 2005). Mass produced sparkling wine typically has shorter aging periods and are often released onto the market shortly afterwards. Sparkling wine production regulations in each country also dictate how soon wine can be released (Pozo-Bayón et al. 2009).

Once the lees aging period has been completed, traditional sparkling wine undergoes three final production steps to produce the finished product. Riddling is the process in which the bottles are tilted at an angle to force the lees into the neck of the bottle. Disgorging is then followed where
the neck of the bottle is frozen, typically in a glycol solution, and lees is removed from wine (Kemp et al. 2017). A small amount of wine may be lost along with the frozen lees when wines are disgorged. A process called liqueur de dosage or liqueur d’expédition also known as dosage is done where a small volume of wine is added to top-up to the desired volume (Pozo-Bayón et al. 2009). The dosage typically consists of the wine itself or another still wine and sometimes a sugar addition to balance the wine and add to the sweetness (Kemp et al. 2017).

1.1.5. Sparkling wine production in cool climate regions

Grapes that are used for wine production are typically grown in the latitudes ranging from 30-50°N to 30-40°S and are favourable in areas that lack extreme heat and cold (Nesbitt et al. 2016). The suitability of the grapes grown in these regions are further determined by local conditions (Nesbitt et al. 2016). Factors such as disease pressure, pests, varietal, climate and weather can affect grapevine development, yield and berry composition (Nesbitt et al. 2016). The effects of climate change, including the increases in temperature and changes in rainfall distribution, may be affecting vine and grape physiology resulting in wine composition and quality (Esteruelas et al. 2015). If ripening temperature is higher than the desired range, grapes mature faster and the resulting pH and sugar concentrations may be too high (Esteruelas et al. 2015). In some wine regions, this has resulted in harvest dates moving forward in recent years (Esteruelas et al. 2015). Wine regions have to adapt wine styles and techniques in coming years to combat the problems that arise from climate change. Research is also suggesting that with climate change, regions situated in higher latitudes, such as cool climate regions, may have increasing viticultural stability (Nesbitt et al. 2016).

Cool climate regions such as the Champagne region in France are known for quality sparkling wine. Sparkling wine produced in Champagne largely contributes to the wine economy and
business in France (Pozo-Bayón et al. 2009). Grapes grown in cool climate regions may not reach full maturity in each growing season resulting in higher acidity and not fully ripened fruit. These grapes are desired in sparkling wine production as they typically have higher titratable acidity (TA), lower pH and lower soluble solids measured in °Brix.

Ontario is a cool climate region and produces similar varietals to those found in Champagne including Chardonnay and Pinot noir (VQA 2015). Riesling, Cabernet franc and Gamay noir also widely grown in Ontario and are considered classic cool climate varietals (VQA 2015). In 2015, it was estimated that the amount used in sparkling wine production was 750 grape tonnage and total grape tonnage was roughly 31,270 (VQA 2015). This amount harvested in the vineyard resulted in 60,738 – 9L cases (VQA 2015). Due to the changing climate and an interest in sparkling wine sales, overall production and the number of wineries producing sparkling wine is constantly increasing. The Vintners Quality Alliance is the governing wine body in Ontario and is responsible for setting wine regulations. It would take approximately a year, from harvest to finished wine, for a winery in Ontario to sell a VQA approved non-vintage dated sparkling wine. VQA Ontario (2015) regulations for traditional method sparkling wine are as follows:

- sparkling wine produced in the traditional method sparkling wine must be made exclusively from *Vitis vinifera* grapes
- vintage-date wine must remain on lees for at least 12 months and non-vintage date wines for a minimum of 9 months
- the finished sparkling wine must be sold in the same bottle in which secondary fermentation took place
1.1.6. Factors affecting sparkling wine quality

Foaming properties, mainly attributed to proteins, in sparkling wine are one of its most important characteristics and indicators of quality (Pozo-Bayón et al. 2009). However, there are other parameters in wine that are responsible for affecting sparkling wine quality which include climate, vineyard conditions, ethanol, acidity, aging time, bentonite addition and CO$_2$ (Cilindre et al. 2014).

1.1.7. Vineyard conditions on sparkling wine quality

Although high yielding vineyards have not been shown to impact foaming properties, they have been shown to have decreased sensory qualities in finished sparkling wine (Pozo-Bayón et al. 2009). Vineyards that are subject to drought stress result in having higher protein concentration in the grapes (Meier et al. 2016). Drought stress can be described as when vines have a physiological response to a water deficit characterized by change in ingredients and growth issues (Meier et al. 2016). The amino acid profiles of wine proteins from drought-stressed grapes are higher than ones without drought-stress (Meier et al. 2016). In still wine production, to prevent protein haze stability, a higher amount of bentonite would be required to remove these proteins (Meier et al. 2016). It is possible that for sparkling wine production, vineyards with drought-stressed grapes would be beneficial for the foaming properties of sparkling wines due to the increased protein concentration.

1.1.8. Aging on sparkling wine quality

Scientists have found that a change in volatile compounds occurs during aging of sparkling wines which can impact quality (Pueyo et al. 1995). Longer lees aging time has been shown to promote different aromatic profiles than no lees or minimum lees aged sparkling wines due to autolysis (Pozo-Bayón et al. 2009). Published research indicates differing results and conclusions regarding whether volatile compounds, such as ethyl esters and acetates, increase or decrease with aging.
Differences may be attributed to the synthesis and degradation of volatile compounds that occur throughout aging with lees and length of time. Cava that was aged for more than 21 months had been found to have increased concentrations of C13 norisoprenoids. This increase was attributed to the enzymes that are released during autolysis of carotenoids (Riu-Aumatell et al. 2006). Furthermore, vitispirane (2,6,6-trimethyl-10-methylidene-1-oxaspiro[4,5]dec-8-ene), a compound described as having “eucalyptus” aromas, has been used as an aging marker in Champagnes as concentrations become apparent in finished and aged sparkling wines (Loyaux et al. 1981). TDN (1,2-dihydro-1,1,6-trimethylnaphthalene), often described as having a “petrol” aroma, is another compound that has been used to differentiate between shorter and longer (<9 months and >20 months respectively) lees aging in Cava (Pozo-Bayón et al. 2009).

1.1.9. Oenological practices on sparkling wine quality

Sparkling wine quality is also impacted by oenological practices, including clarification or filtration, as a reduction in proteins, phenolic compounds and volatile compounds are observed (Pozo-Bayón et al. 2009; Lira et al. 2014). Protein concentration in sparkling wines has been proven to impact wine aromas and quality (Vanrell et al. 2006). Oenological practices such as the use of bentonite to remove proteins negatively impact foam quality in sparkling wine (Pocock et al. 2011). Bentonite is a fining agent that is widely used typically for still wine production to stabilize wines and to prevent haze formation (Jaeckels et al. 2017). Bentonite is also used as a riddling agent in sparkling wine production to help facilitate the flocculation of lees after secondary fermentation (Pozo-Bayón et al. 2009). Bentonite is described as a clay mineral that is characterized by its function as a cation exchanger (Jaeckels et al. 2017). The composition of bentonite consists of approximately 75% montmorillonite which has a multilayer structure of aluminum hydrosilicate forming platelets (Kleijn and Oster 1982). Ca²⁺, Na⁺, or K⁺ are different
cations which are complexed in the interlayer region and have influence in the interlayer distance with absorbing behaviour and swelling (Segad et al. 2010). Unfortunately, bentonite is a non-specific absorber and when it interacts with wine, proteins as well as compounds responsible for aroma and colours also bind and precipitate out of solution (Jaeckels et al. 2017). At wine pH, bentonite is negatively charged and binds to the positively charged proteins (Marangon et al. 2014). The electrostatic interaction between bentonite and proteins in the wine decreases the protein concentration (Vanrell et al. 2006). Different types of bentonite such as sodium bentonite (Na-bentonite) and sodium calcium bentonite (NaCa-bentonite) behave differently in wines as they bind to different groups of proteins (Jaeckels et al. 2017). The timing of bentonite addition and amount of bentonite result in varying foamability behaviour (Lira et al. 2013). The addition of bentonite at large rates can lead to a significant reduction and removal of aroma compounds and a decrease in juice and/or wine volume (Lira et al. 2014). Ethanol has also been shown to have no impact or slightly enhance protein absorption onto bentonite (Achaerandio et al. 2001). This can have downstream effects on final protein concentration and foaming properties in finished sparkling wine.

1.1.10. Fermentation by-products on sparkling wine quality

Carbon dioxide and ethanol are the by-products from secondary fermentation in bottle. The size and number of bubbles are important factors in the sensory evaluation of sparkling wines (Descoins et al. 2004). The ascending velocity and growth rate of bubbles are dependent on the available CO₂ concentration in the liquid phase and by the presence of polysaccharides and proteins on the bubble wall and in the wine (Odake 2001). The presence of ethanol increases the viscosity; however, it does not impact the carbonation concentration (Descoins et al. 2004). An increased level of alcohol, however, has been found to negatively impact foaming. Sugars decrease
the solubility of CO$_2$, while the presence of proteins increase the concentration of CO$_2$ in wine (Descoins et al. 2004).

1.2. Assessing sparkling wine quality

1.2.1. Foam parameters

Sparkling wine quality can be determined chemically, sensorially and by its foaming properties. Although the chemical and sensorial components of sparkling wines are important, the foaming properties in sparkling wine are the driving force in quality as it is what is first introduced to the consumer (Condé et al. 2017). However, to date, there are no legislated standard foaming properties that define sparkling wine quality. From a research perspective, foaming properties are often divided into two categories: foamability and foam stability. The foamability refers to the wine’s ability to form foam, while the foam stability refers to the wine’s ability to maintain the foam or collar (Blasco et al. 2011). Multiple foaming parameters can be measured when evaluating the foaming properties in sparkling wine. These parameters depend on the method used to evaluate the foam and the software available to analyse them. Descriptors such as foam volume, foam height, foam time, collar time, average collar time and average foam lifetime may be used (Lima et al. 2016).

These foaming parameters that can be observed, analyzed and used in determining sparkling wine quality are (Cilindre et al. 2010; Condé et al. 2017; Lima et al. 2016):

- *Foam volume*: the maximum volume of foam formed
- *Foam time*: the duration of the foam before forming the collar
- *Foam height*: the maximum height reached by the foam
• Collar time: length of time where the collar persists (time when the minimum foam height and maximum wine volume has been reached)

• Collar initial height: initial length of the collar throughout the duration of the foam

• Average collar time: average lifetime of the collar

• Average foam lifetime: average lifetime of the foam

• Foam velocity: velocity of dissipation of foam

• Foam expansion: ratio of the foam volume over the time of the maximum height of foam

• % of wine in the foam: percentage of wine present in the foam when maximum foam volume is reached

1.2.2. Foaming assessments

Presently, no internationally standardized method exists to evaluate sparkling wine quality relating to the foaming properties. Additionally, research groups focussing on assessing foaming properties in sparkling wine have developed their own techniques for assessment. Therefore, an absolute standard of ideal foaming properties in quality sparkling wine has not been defined. Examples of methods used to assess foam are the computer assisted viewing equipment (CAVE) method, the Mosalux®, the Rudin tube and the FIZZeyeRobot.

The CAVE technique quantifies foaming properties in sparkling wines while the wine is being poured and after it has been poured (Cilindre et al. 2010). The system has a pouring robot that is assisted by a computer and attached to a data-recording system. The data-recording system collects footage from three video cameras at different angles and has a laser beam that crosses the glass to detect the liquid levels and foam levels as shown in Figure 1.2 (Cilindre et al. 2010). This capability allows for both liquid and foam height to be recorded upon pouring. The resulting data
give information on the maximum foam height, velocities of both the foam and liquid and collar height.

**Figure 1.2:** Setup of CAVE system used to measure the foaming properties in sparkling wines (Cilindre *et al.* 2010).

The Mosalux® technique assesses the foaming properties of any non-effervescent liquids such as sparkling wine that have been degassed or still base wines (Pozo-Bayón *et al.* 2009). It is the most widely used system for determining foaming properties in sparkling wines (Pozo-Bayón *et al.* 2009). The Mosalux® determines the maximum foam height and the foam stability, which is defined by the height at which the foam becomes stable during injection of CO₂ (Cilindre *et al.* 2010). To test the foaming properties in a sparkling wine, wine must be degassed and then poured into a glass cylinder with a glass frit at the bottom. CO₂ is then injected through the frit at a constant pressure and rate. The foam height is then measured during this injection process and afterwards. Figure 1.3 depicts the Mosalux® equipment.
Figure 1.3: Schematic of the equipment to measure the foaming properties in wine using the Mosalux® method: (1) glass tube, (2) thermostatic bath, (3) ultrasonic wave transmitter/receiver, (4) 24 V supply, (5) wave guide, (6) mass flow controller, (7) pressure reducer, (8) regulation valve, and (9) computer (Pozo-Bayón et al. 2009).

The Rudin tube was first developed for testing the foam stability in beer, Figure 1.4 (Rudin 1957). It has since been adapted and used to test foam parameters in sparkling wine (Vincenzi et al. 2014). The foam height of a sparkling wine can be measured when gas flow is introduced into the system followed by measuring the foam decay once gas flow has stopped (Vincenzi et al. 2014). Degassed beer or wine is placed in a glass tube and CO₂ enters the system. The glass tube has gradual measurements and as gas enters through a disc, the resulting foam height can be measured (Rudin 1957).
Figure 1.4: Depicts the Rudin tube setup where tube (A) regulates the pressure during the preparation of the foam, (B) is required when the foam has decayed which gets measured by the selector tap (C) (Rudin 1957).

The FIZZeyeRobot is a newer method used to measure foam quality. Human error during sparkling wine pouring can affect the foaming assessment (Condé et al. 2017). The technique uses a robotic pourer to normalize variability of foam development when sparkling wine is poured. The robotic pourer is attached to a camera that videotapes the pour and assesses multiple parameters including the foamability and bubble count (Condé et al. 2017). This technique is fast and robust, can be used as for quality control purposes and the equipment is portable (Condé et al. 2017). Wine samples do not require degassing prior to analysis. Once the wine has been poured, it is a systematic process to acquiring foaming data (Figure 1.5).
Figure 1.5: FIZZeyeRobot schematic to determine foam parameters (Condé et al. 2017).

If equipment such as the Mosalux® and the FIZZeyeRobot are not available, a technique involving a video camera to measure the foam once poured can be used. The maximum foam height and the foam time can be recorded and analyzed with additional computer software (Curioni et al. 2015). Once sparkling wine is opened, it is poured into a glass sparkling flute or a graduated cylinder and the camera captures the video. This method evaluates finished sparkling wines and samples do not require degassing (Curioni et al. 2015). It is relatively inexpensive technique; however, fewer foam parameters are analyzed and results may vary due to the human error and variability in pouring.
1.3. Role of proteins, grape varietal and yeast on sparkling wine quality

1.3.1. Role of proteins in sparkling wine

Sparkling wine is considered to be a complex matrix due to the abundance of molecules and parameters affecting foam (Vincenzi et al. 2014). Proteins are derived from both grapes and yeast in sparkling wine production and contribute to this complex matrix. Proteins are found in very small concentrations in sparkling wine. They are considered minor components, however, their role in foam stabilization of sparkling wines makes them of utmost importance (Pozo-Bayón et al. 2009). Juice and wine from Vitis vinifera grapes typically contain between 10-500 mg L⁻¹ of protein (Marangon et al. 2014). Some proteins have shown to be good for foaming form or foamability, while others have been shown to promote foam stability (Blasco et al. 2011).

Foam is made up of bubbles and gas that are divided by a thin liquid layer known as the lamellar phase (Blasco et al. 2011). The makeup of foam consists of solids, liquids, gas and surfactants (Blasco et al. 2011). Shown in Figure 1.6, factors such as the amount of foam, size and distribution of the bubbles all determine the foam’s texture. The collapse in foam is attributed to the surface tension that counteracts the forces needed to maintain the foam as foam builds up (Blasco et al. 2011). Surfactants and proteins found in sparkling wine influence foam texture (Pozo-Bayón et al. 2009). Proteins act as macromolecular surfactants, bind to the interface and interact with bubble walls by electrostatic or hydrophobic forces as well as by hydrogen or covalent bonds (Blasco et al. 2011). This binding allows for the formation of a viscoelastic film that is resistant to tension and strong enough to withstand the thickness of the film (Blasco et al. 2011). Thus, proteins are tensioactive as they improve foam stability through film strength and elasticity (Vanrell et al. 2006).
Figure 1.6: (A) Foam structure. (B) Distribution of glycoprotein where the polysaccharides (hydrophilic) are oriented towards the liquid layer while the protein (hydrophobic) is towards the gas bubble (Blasco et al. 2011).

The groups of proteins present in sparkling wine include: invertases, β-glucanases, chitinases and Thaumatin-like proteins (TLP) (Lira et al. 2014). Grape derived proteins typically range from 14-60 kDa in size (Blasco et al. 2011). Proteins in sparkling wine are low molecular weight proteins (Lira et al. 2014). Wine proteins are resistant to low pH and proteolysis.

TLPs and chitinases are known as grape pathogenesis-related (PR) proteins (Marangon et al. 2014). TLPs and chitinases range from 20-30 kDa in size (Meier et al. 2016). These grape proteins are constitutively expressed in healthy plants, as well as expressed in response to abiotic and biotic stress (Marangon et al. 2014). TLPs and chitinases survive vinification; however, over the course of sparkling wine production, both groups of proteins have been found to decrease during fermentation, as well as with the addition of bentonite (Lira et al. 2014; Meier et al. 2016). Invertase, specifically vacuolar invertase, is a grape derived protein that helps with foam stabilization in sparkling wines and has a molecular mass ranging from 60-65 kDa (Blasco et al. 2011). Invertase is one of the most abundant proteins present in wine and is highly hydrophobic.
A decrease in invertase concentration has been found to decrease foam quality in sparkling wines (Dambrouck et al. 2005). Proteins derived from yeast are mainly mannoproteins and proteases. Mannoproteins are released during autolysis. They are classified as glycoproteins and originate from the yeast cell wall (Blasco et al. 2011; Vincenzi et al. 2014). During autolysis, glycoproteins and polysaccharides are hydrolyzed by β-glucanases which release mannoproteins (Martinez-Rodriguez and Pueyo 2009; Blasco et al. 2011). Yeast cell wall composition can be broken down into 85% polysaccharides and 15% proteins. Glycoproteins have been found to be the most prominent macromolecules responsible for foamability and foam stability (Vincenzi et al. 2014). The hydrophobicity of mannoproteins allow them to absorb easily into the gas-liquid interface of foam bubbles. Mannoproteins have several functions including water retention, cell-cell adhesion, biofilm formation, hydrophobicity, enzymatic activity and cell wall porosity (Blasco et al. 2011). The played by mannoproteins on foamability is based on their hydrophobic protein moiety and hydrophilic domains that come in contact with air bubbles and to the aqueous medium. There are two identified yeast proteins that are known to have foamability properties. The first mannoprotein identified from the cell wall of S. cerevisiae sake strain 7 was Awa1p (Shimoi et al. 2002). Fpg1p is the other known protein derived from S. cerevisiae wine strain 145A211 and helps with the foamability in sparkling wines and has a molecular mass of 72.5 kDa (Blasco et al. 2011).

Wine proteins can range from 10-100 kDa in size and typically have a concentration ranging from 4-20 mg L\(^{-1}\) (Blasco et al. 2011). There are many protein determination methods used for juice and wine samples. Colourimetric assays, such as the Bradford assay and the BCA assay, are commonly used. As wine is a complex matrix, components in wine interfere with reagents used in both assays. The Bradford assay is a rapid protein determination technique and it has a short
reaction time. Pectins found in grape juice promote the development of significant coloration in the Bradford assay providing inaccurate protein determinations (Alkorta et al. 1994). Furthermore, ethanol and polyphenols found in wine have been shown to interfere with the Bradford reagents (Marchal et al. 1997; Le Bourse et al. 2010). Reducing sugars such as fructose and glucose, which are predominately found in grape juice, interfere with the BCA assay resulting in falsely elevated protein concentrations (Alkorta et al. 1994). To limit the interference, precipitating the protein from the wine matrix and dissolving the pellet in a buffer or water has been found to eliminate the interference (Gazzola et al. 2012). An emerging technique for protein determination in wine is to use potassium dodecyl sulphate (KDS) to precipitate the protein, then resolubilizing it in water, followed by the BCA assay (Gazzola et al. 2012). Techniques such as sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), fast protein liquid chromatography (FPLC) and reversed phase high performance liquid chromatography (RP-HPLC) have also been used to evaluate protein in wine (Vanrell et al. 2006; Le Bourse et al. 2010).

### 1.3.2. Role of grape varietal and clones in sparkling wine

Grape variety, clone, grape maturity and yield can all impact the final chemical composition of wines, amount of protein and composition resulting in influencing final wine quality and foaming properties (Pozo-Bayón et al. 2009). A study conducted by Fidelibus et al. (2006) on yield components and fruit composition of six different Chardonnay clones found that there are differences among clones when comparing yield components such as cluster weight, berries per cluster and berry weight. These differences were dependent on the vintage year as variation from one year to the following were observed in yield (Fidelibus et al. 2006). However, there was consistency between the magnitude of differences between clones for their yield components (Fidelibus et al. 2006). The chemical composition of the fruit from the clones also varied as one
clone was observed to have higher soluble solids and titratable acidity and lower pH. On the opposite end, another clone was observed to have lower soluble solids, and higher pH (Fidelibus et al. 2006). The clones observed to be lower in soluble solids and higher pH are desired in sparkling wine production. These differences indicate that there is potential for different clones to be ideal for sparkling wine production while others may be more suited for still wine production. Due to the nature of chemical composition required for sparkling wine, the differences in maturity between clones may be a factor used in determining the suitability of clones for production (Fidelibus et al. 2006).

Grape variety can also impact the nitrogen concentrations in the wine. Nitrogen composition in grapes can vary from vintage to vintage. The nitrogen concentration is important as it influences primary and secondary fermentation kinetics as well as the aromatic profiles in the wines (Pozo-Bayón et al. 2009). Some grape varieties are more susceptible to disease and rot in the vineyard. *Botrytis cinerea* is a fungus that can be found on berries and has been shown to impact the protein concentration and composition in the wine (Blasco et al. 2011). Varieties that are susceptible to Botrytis infection are those with tight clusters and thin berry walls where the fungus can spread easily throughout the cluster.

### 1.3.3. Role of yeast in sparkling wine

Yeast selection is very important for the success of sparkling wine production, as yeast go through two fermentations to produce sparkling wine. Yeast enter a low pH, high acidity environment especially during secondary fermentation when the base wine also includes 10-11% (v/v) ethanol and stress responses to these environments are observed. The success of secondary fermentation in bottle is what classifies traditional sparkling wine. There is increasing interest in understanding
how different yeast species and different yeast strains perform during secondary fermentation and the resulting autolysis of yeast during lees aging.

When autolysis of yeast occurs during the aging process, yeast release various peptides, amino acids, proteins and polysaccharides (Blasco et al. 2011). These compounds have a role in foam stabilization and formation (Blasco et al. 2011). Thus, a yeast strain with good autolytic capabilities would be ideal for improving the quality in sparkling wine. Natural autolysis is a slow process and due to the benefits of autolysis there is interest in understanding how to accelerate this process. Presently two methods exist to accelerate autolysis: adding yeast autolysates and increasing the temperature during aging (Pozo-Bayón et al. 2009). However, both methods can potentially lead to off-flavours in the final wine (Charpentier and Feuillat 1993).

Traditionally, commercial Saccharomyces cerevisiae strains are used for secondary fermentation. There is interest in non-S. cerevisiae yeast for sparkling wine production especially in finding yeast that may perform well during autolysis for lees aging, have good flocculation and limit undesirable compounds in sparkling wine. However, there is limited research on non-S. cerevisiae used for secondary fermentation. One yeast, Saccharomyces bayanus – an isolate from Niagara Icewine grapes characterized at CCOVI at Brock University in the Inglis laboratory, referred to as Brock isolate, is of major interest as it has been found to successfully ferment in icewine and appassimento style wines (Heit 2014; Kelly and Inglis 2014). Yeast tolerate high sugar concentrations in icewine and high alcohol in appassimento wine. S. bayanus Brock isolate has also been shown to produce significantly lower levels of undesirable compounds including acetic acid, ethyl acetate and acetaldehyde when compared to S. cerevisiae EC 1118 (Kelly and Inglis 2014). Determining the suitability for the S. bayanus Brock isolate is ideal, as it would help with identifying a signature yeast for Niagara.
Yeast used in sparkling wine production often face low pH, high acidity, sulfur dioxide, low temperature, CO₂ overpressure, high ethanol and low nitrogen environments during certain stages of production (Borrull et al. 2016). Yeast have stress response mechanisms to combat their surrounding environments. Grape juice is a stressful environment for the yeast where, after initial inoculation, yeast experience hypertonic conditions resulting in an efflux of water from the cell, diminished turgor pressure, reduced water availability and cell shrinkage (Bauer and Pretorius 2000). As the strength of this hyperosmotic shock increases, the number of viable yeast cells decreases (Beney et al. 2000). Yeast respond to this condition by modifying the cell wall and cytoskeleton, as well as by synthesizing glycerol to re-establish osmotic equilibrium (Bauer and Pretorius 2000). The synthesis of glycerol is regulated by the high osmolarity glycerol (HOG) signal transduction pathway. The activation of the HOG mitogen-activated protein kinase (MAPK) pathway is another mechanism that yeast undergo in response to hyperosmotic stress and allows for the return to homeostasis.

Once yeast have gone through primary fermentation, the yeast culture in the liqueur de tirage then enters another stressful environment which includes the presence of high ethanol concentrations and high acidity. The ethanol in base wine typically ranges from 10-12%, which can prove to be toxic since ethanol is toxic to many organisms at low concentrations (Bauer and Pretorius 2000). Ethanol affects cell physiology and cellular processes including reduced water activity which affects compartments within the cell (Bauer and Pretorius 2000). Ethanol impacts fermentation efficiency and growth rates due to changes in membrane fluidity and increased membrane permeability (Alexandre et al. 1994). The result in increased permeability leads to the decrease of the proton motive force, which allows for active transport of compounds including amino acids (Bauer and Pretorius 2000).
Yeast’s ability to ferment also depends on nutritional requirements, which vary amongst strains, as well as growing conditions (Bauer and Pretorius 2000). Fermentations may have different nutrient utilisation requirements. Factors such as the presence of other organisms in must and oenological practices, including bentonite fining and clarification prior to fermentation, are influential on yeast growth (Weiss and Bisson 2002). Vineyard conditions, including grape maturity, soil type, viticultural practices and grape variety, influence the grape nutritional composition. If juice is deficient in nitrogen compounds, on which yeast rely during fermentation, supplementation is required to met the nutritional requirements of yeast (Bauer and Pretorius 2000).

1.4. Summary
Sparkling wine is produced in over thirty countries world wide (Robillard and Marchal 2015). There are various methods that are used to make sparkling wine. Traditional sparkling wine production consists of two fermentations. Primary fermentation occurs when the pressed grape juice is inoculated and ferments into base wine. Secondary fermentation is when an active yeast culture is added to the base wine and bottled. Yeast consume sugars and produce ethanol and carbon dioxide. The effervescence is attributed to the carbon dioxide produced from secondary fermentation in bottle (Ribéreau-Gayon et al. 2006).

The effervescence or foam of a sparkling wine is the main driving factor in quality, as it is the first thing consumers notice when opening a bottle. Factors such as climate, grape variety, yeast strain, lees aging and protein affect foaming properties in wine (Cilindre et al. 2014). Champagne, France is considered a cool climate region and has been producing quality sparkling wine for years. Cool climates, including the Niagara Peninsula in Ontario, can benefit from sparkling wine production.
Ontario also produces similar varietals to Champagne, including Chardonnay, Pinot noir and Pinot meunier – three varietals used in Champagne production.

There are various parameters used to evaluate the foaming properties in sparkling wine. Parameters chosen for evaluation depend on the method used to test foam in sparkling wine. Presently, there is no standardized method available globally for evaluating foaming properties. Techniques such as the CAVE method, Mosalux®, Rudin tube and FIZZeyeRobot are commonly used to evaluate foam (Rudin 1957; Vincenzi et al. 2014; Condé et al. 2017).

Traditionally, winemakers use bentonite, a fining agent, to remove unstable proteins. The problem that arises with bentonite usage in sparkling wine production is that it can negatively impact quality in the final wine through altering the protein profile. The electrostatic interaction between bentonite and proteins decreases the protein concentration and in turn affects foaming properties in finished wine (Vanrell et al. 2006; Pocock et al. 2011). Protein concentration also generally decreases throughout the fermentation process (Pocock et al. 2011). Therefore, the rate and timing at which bentonite is added during sparkling wine fermentation affects the final foam quality, since it impacts the protein concentration.

Proteins are a major component in sparkling wine and derive from both grapes and yeast. Some proteins are better foam formers and weak foam stabilizers while other proteins are better foam stabilizers and weak foam formers. Types of proteins found in sparkling wine include invertases, β-glucanases, chitinases and Thaumatin-like proteins (TLP) (Lira et al. 2014). Proteins in sparkling wine are considered low molecular weight proteins (Lira et al. 2014). Wine proteins can range from 10-100 kDa in size and typically have a concentration ranging from 4-20 mg L⁻¹ (Blasco et al. 2011).
Sparkling wine production continues to increase in Ontario. Understanding the best oenological practices including: yeast strain suitability, varietal and clonal composition in grapes, is critical to optimizing sparkling wine production and quality.

1.5. Thesis objectives and hypotheses

The objectives of this project are to:

- Understand the role and relationship that **proteins** have on the overall **foaming properties and overall quality** in sparkling wine produced in Niagara Peninsula, Ontario
- Determine the role different **yeast** and **clones of multiple varietals** have on sparkling wine quality
- Understand how **bentonite** affects sparkling wine quality

Based on the objectives of this project, the first hypothesis was that the presence of grape and yeast proteins will positively affect the foaming properties by promoting better foamability and foam stability. The second hypothesis was that different **Saccharomyces** yeast and different varietals/clones will impact the final chemical composition, foaming properties and overall quality in sparkling wine. Finally, the third hypothesis was that the use of bentonite during sparkling wine production will negatively impact the protein concentration and resulting foaming properties in finished sparkling wine.
1.6. Literature Cited


Heit, C. (2014) Hyperosmotic stress and the impact on metabolite formation and redox balance in *Saccharomyces cerevisiae* and *Saccharomyces bayanus* strains. Brock University, St. Catharines, Canada.


2. The role of grape and yeast proteins on sparkling wine quality produced from Mariafeld Pinot noir and two yeast species

2.1. Abstract

Aims

The objectives of this study are to understand the role that protein concentration has on the foaming properties and overall quality in sparkling wine made from a clone of Pinot Noir, Mariafeld. This study seeks to understand how two yeast species, commercially available *Saccharomyces cerevisiae* EC 1118 and *Saccharomyces bayanus*, isolated from grapes at Brock University, referred to as Brock isolate, have on the overall foaming properties and finished sparkling wine. This study will also determine how the timing of bentonite addition impacts foaming properties in sparkling wine.

Methods and Results

Mariafeld Pinot noir grapes were harvested, whole cluster pressed and the juice was split into two treatments. The control/no treatment juice and a 1.0 g L\(^{-1}\) sodium bentonite (Vitiben) addition was added to the treated juice for removal of grape proteins. The juice was split into four replicates and fermented using a commercial wine yeast, *Saccharomyces cerevisiae* EC1118. Treatments were further divided at secondary fermentation and were either inoculated with *Saccharomyces cerevisiae* EC 1118 or *Saccharomyces bayanus*. For the removal of yeast proteins, 0.95 ml L\(^{-1}\) of liquid sodium bentonite (Inoclar) was added in the tirage. Successful finished sparkling wines were subjected to chemical, protein and foaming analysis. Treatments inoculated with *S. cerevisiae* for secondary fermentation underwent successful fermentation. Treatments inoculated with the *S. bayanus* did not undergo successful secondary fermentation. *S. cerevisiae* EC 1118 treatments
were subjected to chemical, protein and foaming analysis on finished wines. The control/no bentonite treatment had a significantly higher protein concentration in final sparkling wines in comparison to the treatment where both juice and tirage contained bentonite, which had the lowest protein concentration. The control/no bentonite treatment reached the maximum predetermined dissipation of foam time of 10 minutes and had the highest level of foam stability. The wine treated with the most bentonite in the juice and tirage had the lowest level of foam stability.

Conclusions

Mariafeld is a high acid clone of Pinot noir that may be suitable for quality sparkling wine production. *Saccharomyces bayanus* is not a suitable yeast strain for sparkling wine produced from Mariafeld Pinot noir. Factors such as high TA, low pH and alcohol (% v/v) in base wine may have contributed to the lack of viable cells to undergo secondary fermentation in bottle. When using *S. cerevisiae* for primary and secondary fermentation, protein concentration decreases over the course of production and the use of bentonite in secondary fermentation significantly decreases the protein concentration in final sparkling wine. Foaming properties are attributed to grape and yeast proteins. The final foaming properties were negatively impacted by wines that had either grape, yeast or both proteins removed with bentonite.

Significance and Impact

This work provides preliminary insight into understanding how various key factors, such as varietal, yeast species and timing of bentonite addition, affect sparkling wine quality. These findings also show that further understanding of yeast buildup for secondary fermentation needs to be understood, as protocols may be yeast species and/or strain specific so that they are successfully acclimated for bottle fermentation. Furthermore, these results outline issues of protein
determination in wine and ways to overcome these issues. The results from this project provide insight into optimizing sparkling wine parameters to enhance traditional sparkling wine production in the Niagara Peninsula.

**Keywords:** sparkling wine, winemaking, Pinot noir, bentonite, protein, tirage, yeast, *S. cerevisiae*, *S. bayanus*, foamability, SDS-PAGE, Bradford assay, BCA assay

### 2.2. Introduction

Grapes grown in cool climate regions may not reach full maturity in each growing season, resulting in higher acidity and not fully ripened fruit. Although high acidity and low sugar, measured as soluble solids in degrees Brix, may not be optimal for table wine production, these qualities are desired in sparkling wine production. Cool climate regions such as the Niagara Peninsula in Ontario produce similar varietals used in traditional Champagne production including Chardonnay and Pinot Noir (VQAO 2015). As climate continues to change, making it less favourable for sparkling wine production in some areas, regions like Niagara can benefit from sparkling wine production.

Sparkling wine is differentiated from still wine by the foam or effervescence that is formed during the pouring of the wine into a glass (Cilindre *et al.* 2010). Effervescence can be defined as the steady release of dissolved carbon dioxide gas molecules that are responsible for bubble formation (Pozo-Bayón *et al.* 2009).

Foam persistence and stability are two factors which determine the quality of sparkling wine. Various winemaking techniques applied in sparkling wine production can either increase or decrease sparkling wine quality. The addition of bentonite, a clay used as a common fining agent in wine production, at different stages in production impacts the overall foam quality (Lira *et al.*
At wine pH, proteins tend to be positively charged in wine and therefore able to bind to the negatively charged bentonite. The complexed proteins then settle out of the juice or wine and are removed (Marangon et al., 2014). Therefore, bentonite addition impacts protein concentration which, in turn, may impact the overall foaming properties in sparkling wine. Proteins, derived from grapes and yeast, contribute to the foamability in sparkling wine (Pocock et al. 2011).

Factors such as grape variety and yeast strain and protein affect foaming properties in wine (Cilindre et al. 2014). There are currently 43 clones of Pinot noir and it is considered a genetically unstable variety (Hoskins and Thorpe 2010). Scientists believe that between 200-1,000 genetic variants possibly exist in Pinot noir (Hoskins and Thorpe 2010). The parent and offspring have the potential to vary in cluster shape and berry size as well as resulting flavours and aromas (Hoskins and Thorpe 2010). Pinot noir clones picked to be used in sparkling wine production typically have higher acidity, yield, and lower tannins than Pinot noir clones used for table wines (Jones et al. 2014). These clones may also vary in grape protein concentration, which can impact final sparkling wine quality.

*S. cerevisiae* is the predominant yeast strain used in the wine industry. There is interest in using other strains of yeast as populations face environmental stresses throughout fermentation and the capacity to overcome fermentation stresses, such as increased CO$_2$, high ethanol, and low pH environments, is paramount for choosing an appropriate strain in secondary fermentation (Martí-Raga et al. 2015). *S. bayanus* Brock isolate is a yeast strain isolated from Riesling icewine grapes in the Niagara Peninsula known to produce lower levels of oxidative compounds such as acetic acid and ethyl acetate. Understanding the suitability of *S. bayanus* Brock isolate for sparkling wine production is important as it would help with furthering the development of a signature yeast in Niagara wines.
The contribution that grape and yeast proteins have on the foaming properties in Mariafeld Pinot noir will be evaluated, as well as its suitability for quality sparkling wine production. An understanding of how the protein profile and resulting foaming properties in sparkling wine will provide insight into optimizing sparkling wine quality in Niagara, Ontario.

2.3. Materials and Methods

2.3.1. Experimental Design.

![Figure 2.1: Experimental design and breakdown of treatments of sparkling wine produced from Mariafeld Pinot noir.](image)
2.3.2. Yeast strains.

All primary fermentations were completed using commercial yeast *Saccharomyces cerevisiae* Lalvin EC 1118 supplied by Lallemand Inc. (Montreal, QC, Canada). The same yeast (*S. cerevisiae* EC 1118) that was used for primary fermentation was also used for the *S. cerevisiae* treatments in secondary fermentation. The remaining secondary treatments were inoculated with the Brock isolated yeast strain *Saccharomyces bayanus*, which was taken from the bloom of local Riesling icewine grapes (St. Catharines, ON, Canada).

2.3.3. Grape juice.

To investigate the role of proteins on sparkling wine quality, Mariafeld Pinot noir grapes were harvested and whole bunch pressed to 1.1 bar at Trius Winery (Niagara-on-the-lake, ON, Canada). Potassium metabisulfite was added to the juice to give total sulfur dioxide concentration of 50ppm. The juice was then racked into two 200L stainless steel tanks. No further additions were added to the control/no treatment tank. To remove the grape proteins, a 5% w/v sodium bentonite slurry called Vitiben (Bentonite Performance Minerals LLC, Houston, TX, USA) was rehydrated 24 hours in advance and 0.5 g L\(^{-1}\) (g bentonite/L of wine) was added. After settling overnight at 8°C, the juice was racked and transferred to the pilot winery at Brock University. Both juice treatments were then separated into four equal replicates. Initial protein analysis using the Bradford assay determined a lack of removal of grape proteins. An additional 0.5g L\(^{-1}\) of sodium bentonite was added to the bentonite treatment juice. The juice was settled overnight and racked the following day.

2.3.4. Chemical analyses of juice and wine.

Soluble solids of the initial Pinot noir juice were determined with an ABBE bench top refractometer (Model 10450, American Optical, Buffalo, NY, USA). Reducing sugar
concentration was measured per manufacturer’s instructions from a commercial enzyme assay kit (K-FRUGL) (Megazyme International Ireland Ltd; Wicklow, IRE). Juice and wine pH was determined with a Corning pH meter (model 455) with a calibration of pH 4.0, pH 7.0 and pH 10.0. Titratable acidity (g L⁻¹ tartaric acid) was determined by titration with 0.1N NaOH to an endpoint of pH 8.2 (Zoecklein et al. 1996). Yeast assimilable nitrogen content (YANC) is the sum of primary amino nitrogen (PAN) and ammonia nitrogen. PAN and ammonia in the juice and base wine were measured using K-PANOPA chemical assay and K-AMIAR enzymatic kit, respectively (Megazyme International Ireland Ltd; Wicklow, IRE). Malic acid was measured per manufacturer’s instructions from a commercial enzyme assay kit (K-LMAL) (Megazyme International Ireland Ltd; Wicklow, IRE). Free and total sulfur dioxide (SO₂) measurements were performed using aeration-oxidation method on wine samples. Ethanol analysis on wine samples was measured via gas chromatography (Agilent, CA, USA) using an Agilent 6890 model coupled with a flame ionization detector (GC-FID), DB Wax column (30 m x 0.25 mm x 0.25 µm), split/split-less injector and Chemstation software. The pressure of sparkling wines after secondary fermentation was determined using an aphrometer (Afriso Eurogauge Ltd, West Sussex, UK).

**2.3.5. Protein analyses of juice and wine.**

Protein analyses of samples were carried out from the start to finish of sparkling wine production. Initial protein concentration results were determined with the Bradford assay per manufacturer’s instructions (Sigma-Aldrich, St. Louis, MO, USA). Due to pectin interference with the Bradford assay and reducing sugar interference with the BCA assay, samples were subjected to protein precipitation using an acetone precipitation method (Wessel and Flügge 1984). Once precipitation was complete, the precipitated proteins were dissolved in water. The BCA assay was performed on the precipitated proteins from the finished wine samples following the manufacturer’s
instructions (Fisher Scientific, Hampton, NH, USA). Bovine serum albumin (BSA) was used for the calibration curve. Due to the reducing sugar interferences with the BCA assay, even after precipitating the proteins, all samples from every major stage in winemaking were subjected to protein determination using densitometry using SDS-PAGE. For SDS-PAGE analysis, proteins were separated with 12% polyacrylamide gels pH 8.8, 5% stacking gels pH 6.8 and run at 1 hour and 40 minutes at 100V. Gel images were scanned and densitometry was performed using the Bio-Rad Image Lab software (Bio-Rad, Mississauga, ON, CAN).

Total protein in juice or wine was calculated based on the densitometry signal from the SDS-PAGE gels. A 20 µL volume of juice or wine was separated by SDS-PAGE and stained with a SYPRO Ruby stain. Instructions for the SYPRO Ruby stain were followed from manufacturer’s guidelines (Bio-Rad, Mississauga, ON, CAN). A protein ladder (10µL) was also run on each gel in order to act as the reference for protein quantification (Unstained Precision Plus Protein™; Bio-Rad, Mississauga, ON, CAN). The total protein signal from the densitometry signal in each lane from the SDS-PAGE gels was quantified with reference to the densitometry signal for the 50 kDa band (750 ng) when 10 µL of ladder was run on the gel. It was shown that the protein quantification signal from densitometry was linear over a wide range of protein signals (see Appendix I graph A) and that the 50 kDa band in the ladder was a good reference band to use for normalization to control for gel to gel variation in staining (see Appendix I graphs A and B).

The total protein amount (ng) in 20 µL of juice or wine was quantified by comparing the total densitometry signal from each lane to that found for the 50 kDa band of 10 µL of the protein ladder on that same gel. The 50 kDa band represents 750 ng of protein when 10 µL of the ladder is run out on the gel (Unstained Precision Plus Protein™; Bio-Rad, Mississauga, ON, CAN).

Protein concentration in the juice or wine sample (ng/µL) =
(Total protein signal / 50 kDa signal) x (750 ng / 20 µL volume of juice or wine extract)

2.3.6. Sample collection and timepoints.

Juice and wine samples for chemical and protein analyses were collected at every stage of winemaking. The soluble solids, pH, TA, SO₂, Bradford assay and yeast assimilable nitrogen (YAN) analyses were performed on fresh juice or wine samples. The remaining samples for analyses of residual sugar (glucose and fructose), ethanol, malic acid, BCA, SDS-PAGE and ethanol were collected, centrifuged at 10 000 rpm for 5 min to remove grape particulate and yeast cells (Sorvall RC5C Plus, rotor model SLA-3000; Newtown, CT, USA). The samples were then filtered using a 0.45 µm filter pad (Millipore) and aliquotted into 1-250 mL plastic Nalgene container (Thermo-Fisher, Waltham, MA, USA) and 5-1.5mL Eppendorf tubes (Eppendorf Canada, Mississauga, ON, CAN). After centrifugation and filtration samples were frozen at -20°C until analyzed.

2.3.7. Primary fermentation and winemaking.

Primary fermentation of juice to base wine was carried out in eight 60 L plastic containers. The juice was inoculated at a ratio of 0.3 g L⁻¹ and prepared per manufacturer’s instructions (Lallemand Inc, CAN). Go-Ferm® rehydration nutrient (Danstar Ferment A.G., SUI) was added to the rehydration preparation with a ratio of 0.3 g L⁻¹. Nitrogen supplement in the form of diammonium phosphate (DAP) was not added, as sufficient YAN concentration was found in the juice. The plastic fermenters were moved to a fermentation chamber at Brock University that was maintained at 16°C and monitored daily for temperature and sugar consumption. During fermentation, sugar consumption was monitored using a °Brix hydrometer and temperature was monitored using a thermometer labelled in degrees Celsius. The yeast did not appear to be consuming sugar after the first two days of fermentation and the fermenters were then moved to a 20°C chamber for 48 hours,
a 12°C chamber for 24 hours and then back to 16°C chamber for the remainder of fermentation. Primary fermentation was considered complete when the specific gravity was less than 1.000 and showed no further decline for three days. To confirm that fermentations had gone to dryness (less than 5 g L\(^{-1}\) reducing sugar), wine samples were drawn from the fermenters and underwent a FOSS wine scan (WineScan™; Hillerød, DK). Potassium metabisulfite was then added to the base wines at an amount of 40ppm SO\(_2\), blanketed with CO\(_2\) and moved to 4°C. The base wines were racked off their lees, replicates were blended for each treatment and returned to cold storage at -2°C. The free SO\(_2\) levels in the base wines were monitored weekly and to maintain a level of 20ppm SO\(_2\) additions were made accordingly. To help cold stabilize the base wines, potassium bitartrate (Vines to Vintages, Vineland, ON, CAN) was added at a concentration of 4 g L\(^{-1}\) and stirred thoroughly. The base wines were placed at -4°C to cold stabilize and were then racked. For further clarification, the Scottzyme KS enzyme (Scott Laboratories Ltd, CAN), a blended enzyme product of predominately pectinase, was added at room temperature to the base wine at the recommended amount of 0.3 mL gallon\(^{-1}\) (7.93mL L\(^{-1}\)). The base wines were then filtered using a 0.8 μm Seitz KS80 filter pad (Pall Food & Beverage, NY, USA) and then further filtered with a 0.4 μm Fibrafix AF ST 140 filter pad (Filtrox AG, SUI) to have a turbidity reading below 1.5 NTU.

2.3.8. Yeast inoculation procedure for tirage and secondary fermentations.

Four of the eight treatments for secondary fermentation were inoculated with *Saccharomyces cerevisiae* Lalvin EC 1118 (Montreal, QC, CAN) and rehydrated as per manufacturer’s instructions. The remaining treatments to be inoculated with natural yeast strain *Saccharomyces bayanus* Brock isolate were built up in the laboratory. The buildup for *S. bayanus* consisted of plating a frozen culture onto yeast peptone dextrose (YPD media (10 g L\(^{-1}\) yeast extract, 20 g L\(^{-1}\) peptone, 20 g L\(^{-1}\) dextrose and 20 g L\(^{-1}\) agar) and incubated at 30°C. The growth medium to build
up yeast biomass consisted of sterile diluted grape juice to 10ºBrix, diluted with sterile distilled water (RiOs-16; Millipore) with 2 g L\(^{-1}\) of added DAP. One colony from the plated \(S.\) \(bayanus\) was then removed under sterile conditions and added to the growth medium. \(S.\) \(bayanus\) cultures were then placed on a shaker at 25ºC at 130 rpm and allowed to grow aerobically in a sterile environment. The \(S.\) \(bayanus\) was grown to a target concentration like the commercial yeast at approximately 2.2x10\(^9\) cells mL\(^{-1}\). Cell counts were performed under a microscope at 40x magnification with a methylene blue dye to determine the viable and total cell concentration during buildup and throughout tirage using a hemocytometer (Brightline, American Optical Co., Buffalo, NY, USA). Once target concentration of \(S.\) \(bayanus\) culture was reached, it was centrifuged at 3000 rpm for 5 min at 20 ºC (Sorvall RC5C Plus, rotor model SLA-3000; Newtown, CT, USA); supernatant was removed and the precipitated yeast was reconstituted in sterile water. This \(S.\) \(bayanus\) culture was then ready to enter Stage 2 of the tirage buildup process. The tirage build up consisted of three stages. In Stage 1, the commercial \(S.\) \(cerevisiae\) EC 1118 yeast was rehydrated according to manufacturer’s instructions for secondary fermentation at a ratio of 0.1 g L\(^{-1}\). Cultures from Stage 1 were then combined with equal parts base wine and liqueur (base wine with 500 g L\(^{-1}\) added sugar) to put enter Stage 2. These cultures were fermented at the lab bench at 22ºC and were subjected to regular mixing, viable and total cell counts performed with a hemocytometer using methylene blue dye to distinguish viable and non-viable cells and monitoring specific gravity to observe sugar consumption. Once cultures had reached a specific gravity of 1.030, Stage 3 commenced with the further addition of wine, liqueur and water. Specific gravity and cell counts continued throughout this stage. The tirage cultures were ready to be added to the base wine to start secondary fermentation in bottle when a specific gravity of approximately 1.010 was reached. Prior to bottling for secondary fermentation, base wines were then split into four equal replicates.
in the 60 L plastic fermenters for each treatment. For the yeast to undergo successful secondary fermentation, residual sugar was analyzed and base wines received a sucrose addition for a total amount of 24 g L\(^{-1}\) residual sugar. Phosphate titres, a nitrogen supplement with DAP and thiamine was added, at a concentration of 60 mg L\(^{-1}\) (Institute OEnologique de Champagne (IOC), France). Four of the eight treatments then received a liquid sodium bentonite addition (Inoclar 2; IOC, France) at a concentration of 0.95 ml L\(^{-1}\) (approximately 30 mg of bentonite/750 mL bottle). Base wines were then transported to Fielding Estate Winery (Beamsville, ON, CAN) and Stage 3 tirage cultures were added the following day to the base wines. Base wine was mixed, went through the bottling line and was bottled in 750 mL bottles and crown capped. The wines were then labelled, laid on their sides and moved to storage at approximately 13 °C for secondary fermentation and lees aging of 9 months. During secondary fermentation, sparkling wine samples were collected in triplicate for each treatment at time zero (the start of secondary fermentation), half way through lees aging (midpoint) at 5 months and with finished sparkling wine. The samples collected at this timepoint were subjected to chemical and microbial analysis. Microbial analysis consisted of plating 0.1 mL of sparkling wine sample onto yeast peptone dextrose (YPD) plates (10 g L\(^{-1}\) yeast extract, 20 g L\(^{-1}\) peptone, 20 g L\(^{-1}\) dextrose and 20 g L\(^{-1}\) agar) and incubating at 30 °C for 72 hours. Microbial growth was observed after incubation to determine whether autolysis had commenced after 5 months on lees. Furthermore, the midpoint and finished sparkling wine analysis of the bottles included pressure analysis using an aphrometer (Afriso Eurogauge Ltd, West Sussex, UK). After lees aging, bottles of each successful treatment were collected and brought back to Brock University to make the dosage. Sparkling wines were centrifuged at 10 000 rpm for 5 min to remove yeast cells (Sorvall RC5C Plus, rotor model SLA-3000; Newtown, CT, USA) and then filtered using a 0.45 μm filter paper (Millipore; Sigma-Aldrich). Filtered wines were then subject
to a 900 ppm SO\textsubscript{2} and 8 g L\textsuperscript{-1} sucrose addition which created the \textit{dosage}. The \textit{dosage} and remaining bottles from Fielding Estate Winery were then transported to Millesime Sparkling Wine Processing Inc (St. Catharines, ON, CAN) to be riddled and disgorged. Wines were disgorged and a \textit{dosage} of approximately 10 mL was added to each bottle. Bottles were then sealed with a cork and capped with a metal gage. All treatments were then transported back to Brock University for final sparkling wine assessments.

2.3.9. Foaming analysis.

Foaming analysis was filmed using a digital SLR camera with video capability (Canon EOS 70D; Canon Canada Inc., Missisauga, ON, CAN) and took place at Brock University. The temperature of the testing room was at 20ºC and wines ranged from 17ºC - 20ºC. A 250 mL volumetric glass cylinder free of scratches or marks and a timer was used to observe the foaming properties of finished sparkling wine treatments (Curioni \textit{et al.} 2015). Each treatment was assessed in triplicate. After each bottle was opened, a 5-minute wait period ensued to reduce variability between replicates. Once the wait time was over, the video setting was turned on the camera. The bottle was held at 45º and wine was poured into the 250 mL volumetric cylinder. The camera recorded until after all the foam dissipated. The resulting film was then analyzed using Windows Media Player and the total elapsed time for the dissipation of foam and the final wine volume once foam had dissipated was recorded. The remaining wine was then used to chemically assess final wine parameters and for protein analysis.

2.3.10. Statistical analysis.

Differences between variables were determined by the XLSTAT statistical software package released by Addinsoft (Version 7.1; Paris, France). Statistical methods were done using a t-test.
and analysis of variance (ANOVA) with mean separation by Fisher’s Least Significant Difference (LSD; p<0.05).

2.4. Results

2.4.1. Chemical analysis of initial juice and base wine.

The chemical compositions of the Mariafeld Pinot noir control/no treatment juice and bentonite treated juice are given in Table 2.1.

Table 2.1: Chemical composition of Mariafeld Pinot noir prior to fermentation and after primary fermentation of juice to base wine. Values represent the average ± standard deviation of the mean of four replicates analyzed in duplicates (n=8). An asterisk indicates a significant difference (p<0.05) between treatments as determined by a t-test.

<table>
<thead>
<tr>
<th>Pre-inoculated juice</th>
<th>Control/No Treatment</th>
<th>1.0g/L Bentonite Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble Solids (°Brix)</td>
<td>19.1 ± 0.1</td>
<td>18.9 ± 0.1</td>
</tr>
<tr>
<td>Glucose + Fructose (g L⁻¹)</td>
<td>172 ± 5</td>
<td>174 ± 10</td>
</tr>
<tr>
<td>pH</td>
<td>3.08 ± 0.01</td>
<td>3.09 ± 0.01</td>
</tr>
<tr>
<td>Titratable Acidity (g L⁻¹)</td>
<td>13.1 ± 0.0</td>
<td>12.4 ± 0.0</td>
</tr>
<tr>
<td>Amino Nitrogen (mg N L⁻¹)</td>
<td>214 ± 5</td>
<td>210 ± 6</td>
</tr>
<tr>
<td>Ammonia Nitrogen (mg N L⁻¹)</td>
<td>96 ± 0</td>
<td>96 ± 0</td>
</tr>
<tr>
<td>Malic acid (g L⁻¹)</td>
<td>7.5 ± 0.1</td>
<td>7.1 ± 0.1</td>
</tr>
<tr>
<td>Post-ferment base wine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>2.98 ± 0.04</td>
<td>3.03 ± 0.01</td>
</tr>
<tr>
<td>Titratable Acidity (g L⁻¹)</td>
<td>12.1 ± 0.1</td>
<td>12.1 ± 0.1</td>
</tr>
<tr>
<td>Ethanol (% v/v)</td>
<td>11.5 ± 0.1</td>
<td>11.4 ± 0.2</td>
</tr>
<tr>
<td>Glucose + Fructose (g L⁻¹)</td>
<td>0.3 ± 0.0*</td>
<td>0.1 ± 0.0*</td>
</tr>
<tr>
<td>Malic acid (g L⁻¹)</td>
<td>7.3 ± 0.1</td>
<td>7.3 ± 0.1</td>
</tr>
</tbody>
</table>

The chemical composition of the juice after the addition of 1.0 g L⁻¹ sodium bentonite is not significantly different to the control/no bentonite treated juice. The consumption of soluble solids over course of primary fermentation in days can be found in Appendix II, Figure 1.
2.4.2. Liqueur de tirage buildup

The freeze-dried *S. cerevisiae* buildup occurred faster than the live culture buildup of *S. bayanus*. Stage 2 of *tirage* was just under 24 hours for *S. cerevisiae* while *S. bayanus* was in Stage 2 for approximately 32 hours (Tables 2.2 and 2.3).

Table 2.2: Cell viability (%) and specific gravity of *S. cerevisiae* during Stages 2 and 3 of *liqueur de tirage* culture buildup for secondary fermentation in bottle.

<table>
<thead>
<tr>
<th>Stage of tirage</th>
<th>Time in buildup (hours)</th>
<th>Control BW</th>
<th>Bentonite BW</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Specific Gravity</td>
<td>% Viable Cells</td>
<td>Specific Gravity</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>1.052</td>
<td>64.3</td>
</tr>
<tr>
<td>2</td>
<td>10.5</td>
<td>1.034</td>
<td>63.2</td>
</tr>
<tr>
<td>2</td>
<td>23.75</td>
<td>1.010</td>
<td>62.2</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>1.020</td>
<td>58.8</td>
</tr>
<tr>
<td>3</td>
<td>21.5</td>
<td>1.016</td>
<td>55.2</td>
</tr>
<tr>
<td>3</td>
<td>33</td>
<td>1.010</td>
<td>61.4</td>
</tr>
<tr>
<td>3</td>
<td>57.5</td>
<td>1.005</td>
<td>49.8</td>
</tr>
</tbody>
</table>

The % viable cells remains relatively consistent at the end of Stage 2 across the 4 cultures (*S. cerevisiae* Control, *S. cerevisiae* Bentonite, *S. bayanus* Control, *S. bayanus* Bentonite) ranging from approximately 62-72% viable cells (Tables 2.2 and 2.3). At Stage 3, where the culture from Stage 2 is now introduced to an increased ethanol environment as larger volumes of base wine and *liqueur* are added, is where the % viable cells start to steadily decrease in *S. bayanus* cultures. From Table 2.2, the viable cells in both *S. cerevisiae* cultures also decrease over time in Stage 3 but maintain approximately 50+% viability. After just under 60 hours in Stage 3 the *S. cerevisiae* were ready for secondary fermentation in bottle. A rapid decline in viable cells was observed from an earlier *tirage* trial using *S. bayanus* in Stage 3. To minimize this decline Stage 3 was divided into two parts (3a and 3b) for both *S. bayanus* cultures where only half the base wine was incorporated to the culture to decrease ethanol stress response. However, even with only half the
base wine addition, a decrease cell in viability by approximately 20% is observed in both *S. bayanus* cultures (Table 2.3). The decline of viable cells continues from Stages 3a onto 3b resulting in between 35-40% viable *S. bayanus* cells going into secondary fermentation in bottle (Table 2.3).

**Table 2.3:** Cell viability (%) of *S. bayanus* during Stages 2 and 3 of *tirage* culture buildup for secondary fermentation in bottle.

<table>
<thead>
<tr>
<th>Stage of <em>tirage</em></th>
<th>Time in buildup (hours)</th>
<th>Control BW</th>
<th>Bentonite BW</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Specific Gravity</td>
<td>% Viable Cells</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>1.044</td>
<td>57.9</td>
</tr>
<tr>
<td>2</td>
<td>10.5</td>
<td>1.038</td>
<td>57.0</td>
</tr>
<tr>
<td>2</td>
<td>23.75</td>
<td>1.032</td>
<td>75.1</td>
</tr>
<tr>
<td>2</td>
<td>32</td>
<td>1.024</td>
<td>71.6</td>
</tr>
<tr>
<td>3a</td>
<td>0</td>
<td>1.022</td>
<td>73.3</td>
</tr>
<tr>
<td>3a</td>
<td>14.5</td>
<td>1.021</td>
<td>48.4</td>
</tr>
<tr>
<td>3a</td>
<td>20</td>
<td>1.023</td>
<td>49.7</td>
</tr>
<tr>
<td>3b</td>
<td>0</td>
<td>1.023</td>
<td>40.5</td>
</tr>
<tr>
<td>3b</td>
<td>14</td>
<td>1.023</td>
<td>46.1</td>
</tr>
<tr>
<td>3b</td>
<td>17</td>
<td>1.023</td>
<td>43.8</td>
</tr>
<tr>
<td>3b</td>
<td>38.5</td>
<td>1.019</td>
<td>39.4</td>
</tr>
</tbody>
</table>
2.4.3. Chemical analysis of final sparkling wines.

The chemical compositions of the all Mariafeld Pinot noir wine treatments inoculated with *S. cerevisiae* or *S. bayanus* are given in Table 2.4.

**Table 2.4:** Chemical composition of Mariafeld Pinot noir wine treatments. *S. cerevisiae* EC 1118 analysis was performed on final sparkling wine samples while *S. bayanus* samples were analyzed at the lees aging midpoint as secondary fermentation in bottle was not successful. Values represent the average ± standard deviation of the mean of triplicates. Statistical methods used were analysis of variance (ANOVA) with mean separation by Fisher’s Least Significant Difference (LSD; p<0.05). In descending order, lowercase letters indicate statistical difference between treatments at a given timepoint using Fisher’s LDS<sub>0.05</sub>. Due to the successful completion of 4 treatments, the *S. cerevisiae* treatments were statistical compared and the *S. bayanus* treatments were statistical compared separately.

<table>
<thead>
<tr>
<th></th>
<th><em>S. cerevisiae</em> EC 1118</th>
<th><em>S. bayanus</em> Brock isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Bentonite in <em>tirage</em></td>
</tr>
<tr>
<td><strong>Pressure (atm)</strong></td>
<td>5.8 ± 0.3 b</td>
<td>5.9 ± 0.1 ab</td>
</tr>
<tr>
<td><strong>pH</strong></td>
<td>2.99 ± 0.06 a</td>
<td>3.03 ± 0.01 ab</td>
</tr>
<tr>
<td><strong>Titratable Acidity (g L&lt;sup&gt;-1&lt;/sup&gt;)</strong></td>
<td>10.2 ± 0.2 bc</td>
<td>10.1 ± 0.1 c</td>
</tr>
<tr>
<td><strong>Ethanol (% v/v)</strong></td>
<td>11.6 ± 0.0 a</td>
<td>11.3 ± 0.0 b</td>
</tr>
<tr>
<td><strong>Glucose + Fructose (g L&lt;sup&gt;-1&lt;/sup&gt;)</strong></td>
<td>3.5 ± 0.9 b</td>
<td>4.9 ± 0.3 a</td>
</tr>
<tr>
<td><strong>Malic acid (g L&lt;sup&gt;-1&lt;/sup&gt;)</strong></td>
<td>5.7 ± 0.1 b</td>
<td>5.6 ± 0.0 ab</td>
</tr>
<tr>
<td><strong>Pressure (atm)</strong></td>
<td>0.0 ± 0.0 a</td>
<td>0.0 ± 0.0 a</td>
</tr>
<tr>
<td><strong>pH</strong></td>
<td>3.02 ± 0.00 a</td>
<td>3.02 ± 0.01 ab</td>
</tr>
<tr>
<td><strong>Titratable Acidity (g L&lt;sup&gt;-1&lt;/sup&gt;)</strong></td>
<td>10.5 ± 0.03 a</td>
<td>10.4 ± 0.03 ab</td>
</tr>
<tr>
<td><strong>Ethanol (% v/v)</strong></td>
<td>10.6 ± 0.1 a</td>
<td>10.5 ± 0.2 ab</td>
</tr>
<tr>
<td><strong>Glucose + Fructose (g L&lt;sup&gt;-1&lt;/sup&gt;)</strong></td>
<td>26.4 ± 0.7 a</td>
<td>26.6 ± 0.1 a</td>
</tr>
<tr>
<td><strong>Malic acid (g L&lt;sup&gt;-1&lt;/sup&gt;)</strong></td>
<td>5.9 ± 0.3 b</td>
<td>5.9 ± 0.3 b</td>
</tr>
</tbody>
</table>

The chemical analysis of the *S. cerevisiae* EC 1118 treatments was performed on successfully fermented and finished sparkling wine while the *S. bayanus* Brock isolate analysis was performed on the wine samples collected at the 5 month lees aging time point. Initial pressure data of *S. bayanus* treatments at this timepoint show that *S. bayanus* was not able to successfully undergo secondary fermentation in bottle and further chemical analysis was not performed as the final steps...
of sparkling winemaking were not performed. The residual sugar in the *S. bayanus* treatments also indicated a loss of sugar consumption by the yeast from the start of secondary fermentation (Table 2.4). According to *tirage* protocol for secondary fermentation, approximately 24 g L\(^{-1}\) of sugar is added to the base wine prior to bottling for yeast to consume and convert into carbon dioxide and ethanol. No change in residual sugar concentration and ethanol (% v/v) provided further indication of incomplete secondary fermentation by *S. bayanus* treatments. The desired pressure (between 5-6 atm) and ethanol in *S. cerevisiae* treatments was observed in the finished sparkling wine.

### 2.4.4. Protein analysis of juice and wine.

The protein concentration and protein profile of all treatments decrease over the course of production from the initial pressed juice to finished wines. In Figure 2.2, the protein profile of both control/no bentonite and bentonite treated replicates show a decrease in protein from juice treatments prior to primary fermentation to base wine replicates after fermentation.

**Figure 2.2:** Protein profile over the course of primary fermentation of Mariafeld Pinot noir juice to base wine. Lanes: (PMr) Protein Marker, (1) Juice Rep 1, (2) Juice Rep 2, (3) Juice Rep 3, (4) Juice Rep 4, (5) Wine Rep 1, (6) Wine Rep 2, (7) Wine Rep 3, (8) Wine Rep 4 and (9) 25µg ml\(^{-1}\) BSA standard.
Protein bands in juice and wine for both treatments appear around 70 kDa and between 20-37 kDa. There is a significant decrease in protein concentration between juice and base wine for both control and bentonite treatments (Figure 2.3).

**Figure 2.3:** Total protein concentration in pre-inoculated Mariafeld Pinot noir juice and post-fermented base wine determined using densitometry from SDS-PAGE gels. Values represent the average ± standard deviation of the mean of four replicate treatments (n=4). Statistical methods used were analysis of variance (ANOVA) with mean separation by Fisher’s Least Significant Difference (LSD; p<0.05). In descending order, lowercase letters indicate statistical difference between treatments using Fisher’s LDS0.05.
The protein profile of the four control and four bentonite replicates (Figures 2.4A-D) appears to have similar protein bands around 20-35 kDa throughout primary fermentation.

![Protein profile images](image_url)

**Figure 2.4:** Protein profile of control/no treatment and bentonite treated at a given time point in the sparkling wine production of Mariafeld Pinot noir. Lanes: (PMr) Protein Marker, (1) Control rep 1, (2) Control rep 2, (3) Control rep 3, (4) Control Rep 4, (5) Bentonite rep 1, (6) Bentonite rep 2, (7) Bentonite rep 3 and (8) Bentonite rep 4.

The addition of 1.0 g L⁻¹ sodium bentonite appeared to remove grape proteins as a significant different was observed in both juice timepoints (initial pressed juice and post-racked, pre-inoculated juice) between the control and bentonite treatments (Figure 2.5).
**Figure 2.5:** Total protein concentration of control/no treatment and bentonite treatment over four stages in primary fermentation (initial pressed juice, post-racked and pre-inoculated juice, post-fermentation base wine and pre-bottled base wine) determined using densitometry from SDS-PAGE gels. Values represent the average ± standard deviation of the mean of four replicate treatments (n=4). Statistical methods used were analysis of variance (ANOVA) with mean separation by Fisher’s Least Significant Difference (LSD; p<0.05). In descending order, lowercase letters indicate statistical difference between treatments at a given timepoint using Fisher’s LSD0.05.

There was no significant difference between control and bentonite treatment replicates after primary fermentation of the base wine (Figure 2.5). There is however, a significant difference in protein concentration between control and bentonite base wines prior to secondary fermentation in bottle (Figure 2.5). Overall there appears to be a decrease in protein concentration in both control and bentonite treatments over the stages in primary fermentation leading up to secondary fermentation (Figure 2.5).
The protein profile over the course of the three stages in secondary fermentation appears to decrease over time for all treatments tested (Figures 2.6 and 2.7). All treatments appear to have protein bands ranging between 20-25kDa and around 70kDa (Figures 2.6 and 2.7).

Figure 2.6: Protein profile over the course of three timepoints (time zero, lees aging midpoint and finished wine) in secondary fermentation of Mariafeld Pinot noir for all control base wine treatments. Lanes: (PMr) Protein Marker, (1) Time zero rep 1, (2) Time zero rep 2, (3) Time zero rep 3, (4) Midpoint rep 1, (5) Midpoint rep 2, (6) Midpoint rep 3, (7) Final wine rep 1, (8) Final wine rep 2 and (9) Final wine rep 3.
Figure 2.7: Protein profile over the course of three timepoints (time zero, lees aging midpoint and finished wine) in secondary fermentation of Mariafeld Pinot noir for all bentonite base wine treatments. Lanes: (PMr) Protein Marker, (1) Time zero rep 1, (2) Time zero rep 2, (3) Time zero rep 3, (4) Midpoint rep 1, (5) Midpoint rep 2, (6) Midpoint rep 3, (7) Final wine rep 1, (8) Final wine rep 2 and (9) Final wine rep 3.

The control/no bentonite treatment fermented with *S. cerevisiae* in secondary fermentation has the highest protein concentration throughout the course of secondary fermentation and lees aging (Figure 2.8A).
**Figure 2.8:** Total protein profile concentration over the course of secondary fermentation in bottle of Mariafeld Pinot noir base wine to finished sparkling wine (A) inoculated with *S. cerevisiae* EC 1118 (B) inoculated with *S. bayanus Brock isolate* and determined using densitometry from SDS-PAGE gels. Values represent the average ± standard deviation of the mean of triplicates. Statistical methods used were analysis of variance (ANOVA) with mean separation by Fisher’s Least Significant Difference (LSD; p<0.05). In descending order, lowercase letters indicate statistical difference between timepoints over secondary fermentation using Fisher’s LDS$_{0.05}$.

The remaining *S. cerevisiae* treatments (bentonite in juice, bentonite in *tirage*, bentonite in juice & *tirage*) have lower total protein concentrations and are not significantly different from each other in the final sparkling wine (Figure 2.8A). The final timepoint wines for all *S. bayanus*
treatments were opened and analyzed approximately 1 year after all *S. cerevisiae* wines and were stored in a cellar at approximately 16°C throughout this time. Since the *S. bayanus* treatments did not undergo successful fermentation, samples were not collected from these wines until analysis of SDS-PAGE to capture the protein profile in the final wine. There is a decrease in protein concentration over secondary fermentation in both *S. bayanus* control/no bentonite and bentonite in the juice & *tirage* treatments (Figure 2.8B). The bentonite in *tirage* *S. bayanus* treatment shows a decrease in protein from time zero to the lees aging midpoint and then an increase in protein in the final wine (Figure 2.8B). The protein concentration for the bentonite in the juice *S. bayanus* treatment remain stable over the time course of secondary fermentation (Figure 2.8B).
Figure 2.9 depicts the protein profile between treatments with and without the addition of Inoclar 2/liquid sodium bentonite.

**Figure 2.9**: Protein profile of final Mariafeld Pinot noir wine comparing the addition of Inoclar 2/bentonite (IOC, France) in tirage. Lanes: (PMr) Protein Marker, (1) No Inoclar addition rep 1, (2) No Inoclar addition rep 2, (3) No Inoclar addition rep 3, (4) Inoclar addition rep 1, (5) Inoclar addition rep 2, (6) Inoclar addition rep 3, (7) 25µg ml\(^{-1}\) BSA standard.
All treatments with the additional Inoclair 2/liquid sodium bentonite during tirage had a significantly lower protein concentration than the treatments without (Figure 2.10).

**Figure 2.10**: Total protein profile concentration of final Mariafeld Pinot noir wine treatments determined using densitometry from SDS-PAGE gels. Values represent the average ± standard deviation of the mean of triplicates. Statistical methods used were analysis of variance (ANOVA) with mean separation by Fisher’s Least Significant Difference (LSD; p<0.05). In descending order, lowercase letters indicate statistical difference between no Inoclair 2/bentonite addition and Inoclair 2/bentonite addition in tirage between treatments.

The trends when comparing final protein concentration amongst *S. cerevisiae* treatments determined using the BCA assay and densitometry from SDS-PAGE with SYPRO Ruby stain are similar. According to Table 2.5, the control/no bentonite treatment had a significantly higher total protein in the finished sparkling wines compared to the treatments with bentonite in the tirage and with bentonite addition in the juice and tirage. From both methods, there was no significant difference in protein concentration between the three bentonite treatments: in juice, in tirage and in juice and tirage (Table 2.5).
Table 2.5: Protein concentration in finished sparkling wines (S. cerevisiae treatments) determined from BCA assay and densitometry from SDS-PAGE. Analysis was performed in triplicate and samples from each treatment were tested in duplicate for both the BCA assay and densitometry from SDS-PAGE (n=6). Values represent the average ± standard deviation of the mean of triplicates. Statistical methods used were analysis of variance (ANOVA) with mean separation by Fisher’s Least Significant Difference (LSD; p<0.05). In descending order, lowercase letters indicate statistical difference in protein concentration between final sparkling wine treatments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Protein Concentration (µg/mL)</th>
<th>BCA Assay</th>
<th>Densitometry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control/No bentonite</td>
<td>25.2 ± 11.3 a</td>
<td>75.7 ± 11.6 a</td>
<td></td>
</tr>
<tr>
<td>Bentonite in tirage</td>
<td>14.5 ± 5.9 b</td>
<td>58.1 ± 10.8 b</td>
<td></td>
</tr>
<tr>
<td>Bentonite in juice</td>
<td>17.1 ± 10.5 ab</td>
<td>66.0 ± 20.1 b</td>
<td></td>
</tr>
<tr>
<td>Bentonite in juice and tirage</td>
<td>9.9 ± 3.3 b</td>
<td>52.9 ± 5.5 b</td>
<td></td>
</tr>
</tbody>
</table>

The protein profile of the S. cerevisiae final sparkling wines is shown on Figure 2.10. The four treatments have a similar profile of bands with varying signal intensities.

Figure 2.11: Protein profile of all Mariafeld Pinot noir sparkling wine treatments. Lanes: (PMr) Protein Marker, (1) Control BW + S. cerevisiae, (2) Control BW + S. cerevisiae + Inoclar addition, (3) Bentonite BW + S. cerevisiae, (4) Bentonite BW + S. cerevisiae + Inoclar addition, (5) 25µg ml⁻¹ BSA standard.

2.4.5. Foaming analysis of final sparkling wines.

The control/no bentonite treatment has a significantly longer time for the dissipation of foam compared to the bentonite in juice and bentonite in juice and tirage treatments (Figure 2.12A). The bentonite in the juice and tirage treatment took the shortest amount of time for the foam to
dissipate. There was no significant difference in final wine volume from the foaming analysis between the four *S. cerevisiae* treatments (Figure 2.12B).

**Figure 2.12**: Foaming analysis of finished Pinot noir Mariafeld sparkling wine treatments. Values represent the average ± standard deviation of the mean of triplicates. Statistical methods used were analysis of variance (ANOVA) with mean separation by Fisher’s Least Significant Difference (LSD; *p*<0.05). In descending order, lowercase letters indicate statistical difference in protein concentration between final sparkling wine treatments.
2.5. Discussion

2.5.1. Challenges in culture buildup for secondary fermentation

Environmental stresses, including high ethanol, CO₂ concentration, low pH and nitrogen content, are of concern when determining the yeast strain and amounts/types of nutrients required during the acclimation process. The differences in cell viability between the *S. cerevisiae* and *S. bayanus* culture build up are shown in Tables 2.3 and Tables 2.4 respectively. When the *S. bayanus* cultures enter Stage 3 of the *liqueur de tirage* is where we start to see the rapid decline in cell viability. Low concentrations, even as low as 2% (v/v) of ethanol can be toxic to most organisms (Bauer and Pretorius 2000). Furthermore, oxygen requirement is further increased by higher levels of ethanol which continue to increase from primary to secondary fermentation (Bauer and Pretorius 2000). Thus, the increase of ethanol and the lack of oxygen in bottle during secondary fermentation can be detrimental to yeast viability and resulting fermentative capabilities. Appropriate measures during the acclimation of yeast for secondary fermentation are required for a healthy culture to bottle.

The buildup of the *liqueur de tirage* consists of two stages: adaptation and the proliferation phases. During this process, yeast can become acclimatized to the environment and build an appropriate biomass for secondary fermentation. A study by Martí-Raga *et al.* (2015) looked at the role of nitrogen on sparkling wine production. The *liqueur de tirage* culture was built according to the stepwise acclimation for yeast where during the adaptation phase the yeast were cultured in a 6% diluted wine, 50 g L⁻¹ sucrose and 20 mg N L⁻¹ DAP for 48hrs. The ethanol concentration was then increased to 8%, with 50 g L⁻¹ sucrose and additional nitrogen sources during the 96hr proliferation phase. In this study by Martí-Raga *et al.* (2015), the yeast to be used for secondary fermentation
went from a 6% (v/v) to an 8% to a 10% (in base wine) ethanol concentration over time were slowly acclimated to the stressful base wine environments.

According to Table 2.4, *S. bayanus* cultures remained in Stage 2 (a lower % ethanol media) for only 32 hours before entering Stage 3. It is during this switch from Stage 2, the lower ethanol environment, to Stage 3, the higher ethanol environment, where yeast viability is critical. Due to the high ethanol and osmotic stress, a further look into the performance of *S. bayanus* during the *liqueur de tirage* for secondary fermentation needs to be further examined to determine the commercial viability of *S. bayanus* for use in sparkling wine production.

2.5.2. Protein profile and concentration during sparkling wine production

There is a general trend of declining protein concentrations from the beginning to end of sparkling wine production. The highest protein levels are found in the initial juice and drop significantly in both control/no treatment and bentonite treatment. Figures 2.3 and 2.2 show the comparison of treatments before and after primary fermentation. Protein concentrations in *Vitis vinifera* grapes range from 10-500 mg L\(^{-1}\) (Marangon et al. 2014). This trend is similar to that in the literature as scientists have found that protein levels decrease after fermentation (Lira et al. 2014). Figures 2.4 and 2.5 show the decrease in protein over the four sample time points over base wine production including initial pressed juice, post-racked and pre-inoculated juice, post-ferment base wine and pre-bottled base wine. In both control/no treatment and bentonite treatments there is a decline in protein.

The main groups of proteins present in sparkling wine which include invertases, β-glucanases, chitinases and Thuaumatin-like proteins (TLP) (Lira et al. 2014). The proteins in sparkling wine are considered low molecular weight proteins (i.e. invertase at 60kDa and chitinase and TLPs
ranging from 20-30 kDa) (Lira et al. 2014). Although the wine proteins in this study were not further classified, from the profile of the bands in Figures 2.2, 2.6 and 2.7, bands are observed at similar molecular weights, between 20-30 kDa, where we would expect to see chitinases and TLPs. The intensity of the potential invertase bands decrease when comparing juice to final wine as shown in Figures 2.2 and 2.11 respectively. Through SDS-PAGE techniques, scientists have observed the profile of these proteins in selected varietals and their concentration over time. Chitinanse concentrations have been shown to decrease during fermentation, as well as those of TLP decreasing with the addition of bentonite during fermentation (Lira et al. 2014).

The protein levels are further lowered in the final wine, as shown in Table 2.5. Although the BCA assay and densitometry show different concentrations of protein for each treatment, the trends and statistical differences are the same. The control/no bentonite had the highest level of protein as would be expected with no removal of grape and yeast proteins. The three remaining treatments with bentonite (bentonite in the tirage, bentonite in the juice and bentonite in both juice and tirage) show no significant difference in final protein concentration. This suggests that the addition of 1.0 g L⁻¹ sodium bentonite and/or the addition of 0.95 mL L⁻¹ liquid bentonite does not result in a significant difference in the final protein concentration in finished sparkling wine. However, the timing and use of bentonite to strip either grape or yeast proteins appears to play a role in the final foaming properties of sparkling wine.

2.5.3. Foaming properties in finished sparkling wine

The control/no bentonite addition had the highest protein concentration and highest foam persistence of 10 min for the bubbles to fully dissipate (Figure 2.12A). The use of bentonite throughout the winemaking process decreases the protein concentration which appears to negatively impact the foam persistence. Although there was no statistical difference between final
wine volumes (as shown in Figure 2.12B), the control/no bentonite treatment had the lowest final wine volume. This could indicate that more foam and CO₂ was produced upon pouring which resulted in the longest time for the foam to persist.

Furthermore, when autolysis of yeast occurs during the aging process, yeast release various peptides, amino acids, proteins and polysaccharides. These compounds have a role in foam stabilization and formation (Blasco et al. 2011). Mannoproteins are classified as glycoproteins and originate from the yeast cell wall (Blasco et al. 2011; Vincenzi et al. 2014). The hydrophobicity of mannoproteins allows them to absorb easily into the gas-liquid interface of foam bubbles. There was not an observed increase in protein between the zero time point at secondary fermentation through to the protein concentration in the final wine for the control treatment (Figure 2.8A). However, it is possible that autolysis of S. cerevisiae began to occur over the nine-month aging period, releasing mannoproteins that helped with the final foaming properties in the wine. The decline in protein concentration over secondary fermentation could be attributed to a loss of proteins that originated from the grapes during the final processing steps in sparkling wine production. Further research into how the complete removal of grape proteins or presence of grape proteins impacts the foaming properties is suggested to determine their relation to foamability and sparkling wine quality.

2.6. Conclusion

Mariafeld is a high acid clone of Pinot noir and may be suitable for quality sparkling wine production. S. bayanus Brock isolate is not a suitable yeast strain for sparkling wine produced from Mariafeld Pinot noir. Appropriate measures for yeast viability in the liqueur de tirage are necessary for successful fermentation in bottle. Factors such as high TA, low pH and % alcohol in base wine may have contributed to the lack of viable cells to undergo secondary fermentation in bottle.
Protein concentration decreased over the course of sparkling wine production. Grape proteins and yeast proteins are positively contributing factors in the foaming properties in sparkling wine made from Mariafeld Pinot noir.
2.7. Literature Cited


Chapter 3

3. The role of grape and yeast proteins on Riesling sparkling wine quality

3.1. Abstract

Aims

The objectives of this study were to understand the role that protein concentration has on the foaming properties and overall quality in Riesling sparkling wines. The aim was to also determine how grape and/or yeast proteins impact final foaming properties in Riesling sparkling wine.

Methods and Results

Riesling grapes were harvested and the juice was split into three treatments: the control/no treatment; a pectinase treated juice; and a 2.0 g L\(^{-1}\) sodium bentonite (Bentogram\(^{®}\)) and pectinase treated juice. Each treatment was split into three replicates and primary fermentation was completed with a commercial wine yeast, *Saccharomyces cerevisiae* EC 1118. Once fermented, the replicates were blended and base wine underwent cold stabilization and filtration. All three treatments were then further inoculated with the same *S. cerevisiae* EC 1118 yeast for secondary fermentation. Each of the three treatments were further divided at the tirage stage with either no further bentonite addition or the addition of 0.95 ml L\(^{-1}\) liquid sodium bentonite (Inoclair 2) for the removal of yeast proteins. Finished sparkling wines were subjected to chemical, protein and foaming analyses. The control/no bentonite treatments and the pectinase treatments had a higher protein concentration in final sparkling wines than the treatments treated with sodium bentonite at the initial juice stage. Due to a large amount of gushing of the wines at the disgorging and dosage stage of processing, pressure and foam was lost in the final wines across all treatments. Foaming
analysis was completed on all finished wines; however no treatments could retain a high level of foaming, with the longest treatment having a dissipation time for foam of 42 seconds.

**Conclusions**

The addition of 2.0 g L\(^{-1}\) of sodium bentonite completely stripped the grape proteins from the bentonite treated juice. During the *tirage* buildup, yeast biomass increases and releases proteins in the wine, as an increase in protein was observed at the start and throughout secondary fermentation for the juice bentonite treatments that had no grape protein present but which showed protein after yeast addition. The riddling process is very important in production, as lees that does not settle in the neck of the bottle may create nucleation points and increase gushing, accompanied by a loss of wine, resulting in a decrease in final wine pressure and less foam. Due to the extensive gushing across all treatments at the disgorging and dosage stage, no conclusions could be drawn about the role of Riesling grape proteins on its own or in combination with yeast proteins on finished sparkling wine foaming properties. The use of Riesling grapes along with appropriate oenological practices should be further researched to determine the suitability of Riesling for high quality traditional method sparkling wines in the Niagara Peninsula, Ontario.

**Significance and Impact**

This work provides an understanding into the impact of bentonite addition at the juice stage and *tirage* stage and its effect on final chemical composition and protein concentrations in sparkling wine. These results give further understanding of protein interactions and the role of protein in sparkling wine.

**Keywords:** sparkling wine, winemaking, Riesling, bentonite, protein, *tirage*, yeast, *S. cerevisiae*, foaming properties, riddling, gushing, disgorging, dosage, SDS-PAGE, BCA assay
3.2. Introduction

Riesling is a varietal from the *Vitis vinifera* grape species and is widely grown in cool climate regions. Aromas that are typically used to describe wines made with Riesling grapes include: citrus, floral, peach and petrol (VQAO 2015). Although Riesling is not considered one of the three varietals used in traditional Champagne, it can still be used in sparkling wine production in other parts of the world outside of the Champagne region of France. The Niagara Peninsula, Ontario is a source of premium wines in Canada and is constantly expanding its wine production (VQAO 2015). Riesling is also one of the more commonly grown grapes in the Niagara Peninsula wine region of Ontario (VQAO 2015). Riesling grapes are typically higher in acidity and may be suitable for quality sparkling wine production. With ongoing climate change and an increased growth in the market for sparkling wine (VQAO 2013), understanding the suitability of Riesling for sparkling wine production may prove to be beneficial.

Quality sparkling wine is often described as wines that are good foam formers and can maintain the foam once poured. The foaming properties in sparkling wines are the first identifying factors of quality to the consumer (Pozo-Bayón *et al*. 2009). Other factors, such as aroma and flavour, are also important in defining quality sparkling wine. The terms foamaibility (ability to form foam) and foam stability (ability to maintain the foam) are what are often used to determine the quality of sparkling wine (Blasco *et al*. 2011). These foaming properties can be measured in various ways, including determining foaming parameters by testing base wines as well as finished sparkling wines.

Vineyard practices, such as irrigation, pest management and leaf removal, impact the health and chemical composition of grape berries (Kemp *et al*. 2017). Appropriate vineyard practices during the growing season should be applied to maintain the quality of fruit for wine production. Once
grapes have been harvested, winemakers make multiple decisions at each stage in sparkling wine production that may eventually determine the final foaming properties in sparkling wine. Practices such as the addition of clarification enzymes in the juice to breakdown grape pectins, addition of bentonite in the juice to further clarify the juice, bentonite addition in the tirage during the bottle fermentation to assist in yeast settling, and the amount of time on lees can all affect the quality of sparkling wine (Kemp et al. 2017).

The addition or omission of the previously mentioned practices affect the protein composition in grape juice and wine. These wine proteins, which are derived from both grapes and yeast during sparkling wine production, can play a role in the foaming properties in finished wine (Vanrell et al. 2006). Types of proteins found in wine include Thaumatin-like proteins (TLPs), chitinases, β-glucanases and invertases (Rowe et al. 2010; Vincenzi et al. 2010 Lira et al. 2014). TLPs, chitinases and invertases are grape-derived and are known for causing protein instability in table wines (Vincenzi et al. 2010). The presence of β-glucanases in sparkling wine may increase the aging characteristics of the wine (Rodríguez-Nogales et al. 2012). Grape invertases convert sucrose into fructose and glucose and are considered a major protein in wine regardless of grape variety (Cilindre et al. 2014). TLPs accumulate during fruit ripening (Cilindre et al. 2014). Concentrations of these low molecular weight grape proteins typically decrease after fermentation (Lira et al. 2014). Furthermore, the addition of bentonite favours the removal of lower molecular weight proteins including TLPs over wine proteins (Cilindre et al. 2014). In addition to these classes of protein all found in still and sparkling wines, mannoproteins are glycosylated proteins that are derived from yeast and can also be present in both base wine and sparkling wines. Mannoproteins increase the foamability of sparkling wines. Increased lees aging may increase the
concentration of mannoproteins in finished sparkling wine and improve the foaming properties (Rowe et al. 2010).

This research is part of a larger project that aims to investigate the role of protein in finished sparkling wine and its effect on foamability. The objective of this study was to evaluate the suitability of Riesling, a high acid grape, for quality sparkling wine production and the role of grape- and yeast-derived proteins in foamability and foam stability.

3.3. Materials and Methods

3.3.1. Experimental Design.

**Grape Varietal**
- Riesling

- Control/No Treatment
  - Control Base Wine
    - Control
    - Control + Inoclair
  - Pectinase Treated Juice
    - Pectinase
    - Pectinase + Inoclair
  - Pectinase & Bentonite (2.0 g/L) Treated Juice
    - Pectinase + Bentonite
    - Pectinase + Bentonite + Inoclair

**Figure 3.1:** Experimental design and breakdown of treatments of sparkling wine produced from Riesling grapes.
3.3.2. Yeast.

Primary and secondary fermentations were completed using commercial yeast *Saccharomyces cerevisiae* Lalvin EC 1118 supplied by Lallemand Inc. (Montreal, QC, Canada).

3.3.3. Grape juice.

To investigate the role of proteins on sparkling wine quality, Riesling grapes from the Niagara Peninsula were harvested and whole bunch pressed in a bladder press to 1.5 bar in the Pilot Winery at Brock University (St. Catharines, ON, Canada). SO₂ was added to the pressed juice at a concentration of 50 ppm total sulfur dioxide and juice was separated into nine 11.5 L plastic carboys as the three treatments were separated into triplicates. The control treatment received no additional treatment. Pectinase (LAFAZYM®, Laffort, Bordeaux, France) was added to one of the treated juices at the manufacturer’s suggested amount (5 mg L⁻¹). To completely remove grape proteins, a Scottzyme KS enzyme (Scott Laboratories Ltd, CAN), a blended enzyme product with predominately pectinase, along with a 5% w/v sodium bentonite slurry called Bentogram® (AEB USA, Lodi, CA, USA) was added at a concentration of 2 g L⁻¹ (g bentonite L⁻¹ of juice). All juice treatments were then allowed to settle at 4°C for 72 hours. After settling, the juice was racked and initial protein analysis using the Bradford assay and SDS-PAGE with SYPRO Ruby stain was conducted to confirm the removal of grape proteins in the bentonite treatment.

3.3.4. Chemical analyses of juice and wine.

Soluble solids of the initial Riesling juice were determined with an ABBE bench top refractometer (Model 10450, American Optical, Buffalo, NY, USA). Reducing sugar concentration (glucose and fructose) was measured per manufacturer’s instruction from a commercial enzyme assay kit (K-FRUGL) (Megazyme International Ireland Ltd; Wicklow, IRE). Juice and wine pH was determined with a Corning pH meter (model 455) with a calibration of pH 4.0, pH 7.0 and pH
Titratble acidity (TA g L\(^{-1}\) tartaric acid) was determined by titration with 0.1 N NaOH to an endpoint of pH 8.2 (Zoecklein et al. 1996). Yeast assimilable nitrogen content (YANC) is the sum of primary amino nitrogen (PAN) and ammonia nitrogen. PAN and ammonia in the juice and base wine was measured using K-PANOPA chemical assay and K-AMIAR enzymatic kit, respectively (Megazyme International Ireland Ltd; Wicklow, IRE). Malic acid was measured per manufacturer’s instructions from a commercial enzyme assay kit (K-LMAL) (Megazyme International Ireland Ltd; Wicklow, IRE). Free and total sulfur dioxide (SO\(_2\)) measurements were performed using aeration-oxidation method on wine samples. Ethanol analysis on wine samples was measured via gas chromatography (Agilent, CA, USA) using an Agilent 6890 model coupled with a flame ionization detector (GC-FID), DB Wax (30 m x 0.25 mm x 0.25 μm), split/split-less injector and Chemstation software. The pressure of sparkling wines after secondary fermentation was determined using an aphrometer (Afriso Eurogauge Ltd, West Sussex, UK).

3.3.5. Protein analyses of juice and wine.

Protein analyses of samples were carried out from the start to finish of sparkling wine production. Initial protein concentration results were determined with the Bradford assay per manufacturer’s instructions (Sigma-Aldrich, St. Louis, MO, USA). Due to pectin interference with the Bradford assay and reducing sugar interference with the BCA assay, samples were subjected to protein precipitation using an acetone precipitation method (Wessel and Flügge 1984). Once precipitation was complete, the precipitated proteins were dissolved in water. The BCA assay was performed on the precipitated proteins from the finished wine samples following the manufacturer’s instructions (Fisher Scientific, Hampton, NH, USA). Bovine serum albumin (BSA) was used for the calibration curve. Due to the reducing sugar interference with the BCA assay, even after precipitating the proteins, predominately in juice, all samples from every major stage in
winemaking were subjected to protein determination using densitometry using SDS-PAGE. For SDS-PAGE analysis, proteins were separated with 12% polyacrylamide gels pH 8.8, 5% stacking gels pH 6.8 and run at 1 hour and 40 minutes at 100V. Gel images were scanned and densitometry was performed using the Bio-Rad Image Lab software (Bio-Rad, Mississauga, ON, CAN).

Total protein in juice or wine was calculated based on the densitometry signal from the SDS-PAGE gels. A 20 µL volume of juice or wine was separated by SDS-PAGE and stained with a SYPRO Ruby stain. Instructions for the SYPRO Ruby stain were followed from manufacturer’s guidelines (Bio-Rad, Mississauga, ON, CAN). A protein ladder (10µL) was also run on each gel in order to act as the reference for protein quantification (Unstained Precision Plus Protein™; Bio-Rad, Mississauga, ON, CAN). The total protein signal from the densitometry signal in each lane from the SDS-PAGE gels was quantified with reference to the densitometry signal for the 50 kDa band (750 ng) when 10 µL of ladder was run on the gel. It was shown that the protein quantification signal from densitometry was linear over a wide range of protein signals (see Appendix I graph A) and that the 50 kDa band in the ladder was a good reference band to use for normalization to control for gel to gel variation in staining (see Appendix I graphs A and B).

The total protein amount (ng) in 20 µL of juice or wine was quantified by comparing the total densitometry signal from each lane to that found for the 50 kDa band of 10 µL of the protein ladder on that same gel. The 50 kDa band represents 750 ng of protein when 10 µL of the ladder is run out on the gel (Unstained Precision Plus Protein™; Bio-Rad, Mississauga, ON, CAN).

Protein concentration in the juice or wine sample (ng/µL) =

(Total protein signal / 50 kDa signal) x (750 ng / 20 µL volume of juice or wine extract)
3.3.6. Sample collection and timepoints.

Juice and wine samples for chemical and protein analyses were collected at every stage of winemaking. The soluble solids, pH, TA, SO₂, Bradford assay and YAN analyses were performed on fresh juice and wine samples. The remaining samples for analyses of residual sugar (glucose and fructose), ethanol, malic acid, BCA, SDS-PAGE and ethanol were collected, centrifuged at 10,000 rpm for 5 min to remove grape particulate and yeast cells (Sorvall RC5C Plus, rotor model SLA-3000; Newtown, CT, USA). The samples were then filtered using a 0.45 μm filter pad (Millipore, Sigma-Aldrich) and aliquoted into 1-250 mL plastic Nalgene container (Thermo-Fisher, Waltham, MA, USA) and 5-1.5 mL Eppendorf tubes (Eppendorf Canada, Mississauga, ON, CAN). After centrifugation and filtration, samples were frozen at -20°C until analyzed.

3.3.7. Primary fermentation and winemaking.

Primary fermentation of juice to base wine was carried out in nine 11.5 L plastic carboys. The juice was inoculated at 0.3 g L⁻¹ and prepared per manufacturer’s instructions (Lallemand Inc, CAN). GoFerm® rehydration nutrient (Danstar Ferment A.G., CH) was added to the rehydration preparation at 0.3 g L⁻¹. Nitrogen supplement in the form of diammonium phosphate (DAP) was added to target 275 mg N L⁻¹, as insufficient YAN concentration was found in the Riesling juice to fully support the yeast growth required for fermentation. The plastic fermenters were moved to a fermentation chamber at Brock University that was maintained at 18°C for the first 48 hours and then reduced to 16 °C for the remainder of fermentation. Carboys were monitored daily for temperature and sugar consumption. During fermentation, sugar consumption was monitored using a ºBrix hydrometer and temperature was monitored using a thermometer labelled in degrees Celsius. Primary fermentation was considered complete when the specific gravity was less than 1.000 and showed no further decline for three days. To confirm that fermentations had gone to
dryness (less than 5 g L\(^{-1}\) reducing sugar), wine samples were initially drawn from the fermenters and underwent sugar quantification using a FOSS wine scan (WineScan\(^{\text{TM}}\); Hillerod, DK) and quantified using the residual sugar (glucose + fructose) enzyme assay kit (K-FRUGL) (Megazyme International Ireland Ltd; Wicklow, IRE). Potassium metabisulfite was then added to the base wines at a concentration of 40 ppm SO\(_2\), blanketed with CO\(_2\) and moved to 4\(^\circ\)C. The base wines then were racked off their lees, replicates were blended for each treatment and returned to cold storage at -2\(^\circ\)C. The free SO\(_2\) levels in the base wines were monitored weekly and, to maintain a level of 20ppm SO\(_2\), additions were made accordingly. To help cold stabilize the base wines, potassium bitartrate (Vines to Vintages, Vineland, ON, CAN) was added at 4 g L\(^{-1}\) and stirred thoroughly. The base wines were placed at -4 \(^\circ\)C to cold stabilize and were then racked. The base wines were then filtered using a 0.8 \(\mu\)m Seitz KS80 filter pad (Pall Food & Beverage, NY, USA) in order to reach a turbidity reading below 1.5 NTU. Due to ethanol levels in the Riesling base wines coming in above 11\% (v/v), the base wines were diluted 1.12-fold with Milli-RiOs water (RiOS-16; Millipore, Etobicoke, ON, CAN) to decrease the ethanol concentration to average 11.0 \% (v/v) ethanol. With the dilution, sucrose additions for secondary fermentation in bottle allowed for approximately 1.5-2\% increase in finished sparkling wine.

3.3.8. Yeast inoculation procedure for tirage and secondary fermentations.

All treatments for secondary fermentation were inoculated with Saccharomyces cerevisiae Lalvin EC 1118 (Montreal, QC, CAN). The tirage build up consisted of three stages. In Stage 1, the commercial S. cerevisiae EC 1118 yeast was rehydrated according to manufacturer’s instructions for secondary fermentation at 0.1 g L\(^{-1}\). Cultures from Stage 1 were then combined with equal parts base wine and liqueur (base wine with 500 g L\(^{-1}\) added sugar) to enter Stage 2. These cultures were fermented at the lab bench at 22\(^\circ\)C and were subjected to regular mixing, cell counts with a
hemocytometer and monitoring of specific gravity to observe sugar consumption. Once cultures had reached a specific gravity of 1.030, Stage 3 commenced with the further addition of wine, liqueur and water. Specific gravity and cell counts continued throughout this stage. The *tirage* cultures were ready to be added to the base wine when a specific gravity range of 1.000-1.010 was achieved to start secondary fermentation in bottle. The initial three *tirage* cultures from this buildup did not appear to consume sugars, as the specific gravity remained unchanged and viable cell counts decreased over time. A new *tirage* culture was built up by increasing the amount of yeast to 0.15 g L⁻¹, addition of 175 mg L⁻¹ of Go-Ferm® in Stage 1, and diammonium phosphate (DAP) was added to the wine at 175 mg L⁻¹ in Stages 2 and 3.

To undergo secondary fermentation, residual sugar was analyzed and base wines received a sucrose addition for a total amount of 24 g L⁻¹ residual sugar for yeast to consume. Diammonium phosphate (DAP) was also added to the base wine at 175 g L⁻¹ to help the yeast ferment. Bottling for secondary fermentation took place in the Pilot Winery at Brock University. Each *tirage* culture was based on a 60 L wine volume. According to this, the volume of *tirage* culture for a 750 mL bottle ferment was calculated to be 22.5 mL. Approximately 727.5 mL of base wine from each treatment was first measured out in a 1000 mL plastic graduated cylinder and poured using a funnel into the bottle. Then 22.5 mL of the *tirage* culture was added using the automatic pippetter (Eppendorf, Mississauga, ON, CAN). The bottle was then crown capped, labelled and placed on its side in the cage. Half of the three different treatments were bottled first. The remaining half of the three treatments then received a liquid sodium bentonite addition (Inoclair 2; IOC, France) at 0.95 mL L⁻¹ and then also received 22.5 mL of *tirage* culture, crown capped, labelled and placed on its side in the cage. Bottled wines were then transported to Fielding Estate Winery (Beamsville,
ON, CAN) and stored at approximately 13 °C for secondary fermentation and lees aging of 9 months.

During secondary fermentation, sparkling wine samples were collected in triplicate for each treatment at time zero (the start of secondary fermentation), half way through lees aging (midpoint) at 5 months and for finished sparkling wine. The samples collected at the midpoint timepoint were subjected to chemical and microbial analysis. Microbial analysis consisted of plating 0.1 mL of sparkling wine sample onto yeast peptone dextrose (YPD) plates (10 g L⁻¹ yeast extract, 20 g L⁻¹ peptone, 20 g L⁻¹ dextrose and 20 g L⁻¹ agar) and incubating at 30°C for 72 hours, as well as assessing viable cell counts using a hemocytometer. Microbial growth was observed after incubation to determine whether autolysis had commenced after 5 months on lees. Furthermore, the midpoint and finished sparkling wine analysis of the bottles included pressure analysis using an aphrometer (Afriso Eurogauge Ltd, West Sussex, UK).

After lees aging, three bottles of each successful treatment were collected and brought back to Brock University to make the dosage. These bottles of sparkling wines from each treatment were centrifuged at 10 000 rpm for 5 min to remove yeast cells (Sorvall RC5C Plus, rotor model SLA-3000; Newtown, CT, USA), filtered using a 0.45 μm filter paper (Millipore, Sigma-Aldrich) and then pooled together. These filtered wines for each treatment were then subjected to a 900 ppm SO₂ addition to create the dosage wine to be used for the dosage addition. The dosage and remaining bottles from Fielding Estate Winery were then transported to Millesime Sparkling Wine Processing Inc (St. Catharines, ON, CAN) to be riddled and disgorged. The remaining bottles of sparkling wines were disgorged and a dosage of approximately 10 mL was added to each bottle. Bottles were then sealed with a cork and capped with a metal cage. All treatments were then transported back to Brock University for final sparkling wine assessments.
3.3.9. Foaming analysis.

The foaming analysis of finished sparkling wine treatments took place 8 weeks after the final wines underwent disgorging and dosage additions. Foaming analysis was filmed using a digital SLR camera with video capability (Canon EOS 70D; Canon Canada Inc., Mississauga, ON, CAN) and took place at Brock University. The temperature of the testing room was at 20°C and wines ranged from 17°C - 20°C. A 250 mL volumetric glass cylinder free of scratches or marks and a timer was used to observe the foaming properties of finished sparkling wine treatments (Curioni et al. 2015). Each treatment was assessed in triplicate. After each bottle was opened, a 5-minute wait period ensued to reduce variability between replicates. Once the wait time was over, the video setting was turned on the camera. The bottle was held at 45° and wine was poured into the 250 mL cylinder. The camera recorded until after all the foam dissipated. The resulting film was then analyzed using Windows Media Player and the total elapsed time for the dissipation of foam and the final wine volume once foam had dissipated was recorded. The remaining wine was then used to chemically assess final wine parameters and for protein analysis.

3.3.10. Statistical analysis.

Differences between variables were determined by the XLSTAT statistical software package released by Addinsoft (Version 7.1; Paris, France). Statistical methods were analysis of variance (ANOVA) with mean separation by Fisher’s Least Significant Difference (LSD; p<0.05).

3.4. Results

3.4.1. Chemical analysis of initial juice and base wine.

The chemical compositions of the three Riesling treatments before and after primary fermentation are given in Table 3.1: control/no treatment; pectinase treated; and bentonite treated.
Table 3.1: Chemical composition of Riesling juice after treatment and prior to and after primary fermentation into the base wine. Values represent the average ± standard deviation of the mean of triplicates analyzed in duplicates (n=6). Statistical methods used were analysis of variance (ANOVA) with mean separation by Fisher’s Least Significant Difference (LSD; p<0.05). In descending order, lowercase letters indicate statistical difference between treatments using Fisher’s LDS0.05.

<table>
<thead>
<tr>
<th></th>
<th>Control/No Treatment</th>
<th>Pectinase Treatment</th>
<th>Bentonite Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble Solids (ºBrix)</td>
<td>20.9 ± 0.1 a</td>
<td>20.9 ± 0.1 a</td>
<td>20.2 ± 0.1 b</td>
</tr>
<tr>
<td>Glucose + Fructose (g L⁻¹)</td>
<td>232 ± 5 a</td>
<td>235 ± 2 a</td>
<td>215 ± 2 b</td>
</tr>
<tr>
<td>pH</td>
<td>2.99 ± 0.01 a</td>
<td>2.99 ± 0.01 a</td>
<td>2.99 ± 0.00 a</td>
</tr>
<tr>
<td>Titratable Acidity (g L⁻¹)</td>
<td>7.9 ± 0.0 a</td>
<td>7.2 ± 0.0 b</td>
<td>6.8 ± 0.0 c</td>
</tr>
<tr>
<td>Amino Nitrogen (mg N L⁻¹)</td>
<td>65 ± 0 a</td>
<td>67 ± 1 a</td>
<td>61 ± 0 b</td>
</tr>
<tr>
<td>Ammonia Nitrogen (mg N L⁻¹)</td>
<td>24 ± 1 a</td>
<td>23 ± 0 a</td>
<td>23 ± 1 a</td>
</tr>
<tr>
<td>Malic acid (g L⁻¹)</td>
<td>1.5 ± 0.0 b</td>
<td>1.7 ± 0.0 a</td>
<td>1.6 ± 0.0 a</td>
</tr>
</tbody>
</table>

The chemical composition of the control/no treatment and pectinase treated juice are not significantly different across all parameters except titratable acidity and malic acid. The bentonite treated juice however, has significantly lower soluble solids, sugars (glucose and fructose), titratable acidity, and amino acids when compared to the other two treatments. The addition of 2.0 g L⁻¹ sodium bentonite, along with the KS pectinase enzyme followed by racking the juice, appears to have removed some soluble solids in solution as well as dropping acids and amino acids. The consumption of soluble solids over the course of primary fermentation in days can be found in Appendix III, Figure 1. The control treatment starts off slower than the pectinase and bentonite treated juices but all three treatments reach completion by day 15. After primary fermentation, the control/no treatment and the pectinase treatment have a significantly higher ethanol (% v/v)
concentration than the bentonite treatment. The residual sugar is also significantly lower in the control/no treatment.

3.4.2. Liqueur de tirage.

The tirage live culture buildup with freeze-dried *S. cerevisiae* EC 1118 is given in Table 3.2.

Table 3.2: Cell viability (%) and specific gravity of *S. cerevisiae* during Stages 2 and 3 of tirage culture buildup for secondary fermentation in bottle. Cell viability (%) and specific gravity of *S. cerevisiae* during Stages 2 and 3 of tirage culture buildup for secondary fermentation in bottle with the addition of 175 mg L\(^{-1}\) of Go-Ferm® in Stage 1 and 175 mg L\(^{-1}\) of DAP in Stages 2 and 3.

<table>
<thead>
<tr>
<th>Stage of tirage</th>
<th>Time in buildup (hours)</th>
<th>Control BW</th>
<th>Pectinase BW</th>
<th>Bentonite BW</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Specific Gravity</td>
<td>% Viable Cells</td>
<td>Specific Gravity</td>
</tr>
<tr>
<td>2</td>
<td>0.5</td>
<td>1.066</td>
<td>82.7</td>
<td>1.065</td>
</tr>
<tr>
<td>2</td>
<td>19.5</td>
<td>1.034</td>
<td>76.6</td>
<td>1.032</td>
</tr>
<tr>
<td>2</td>
<td>21.5</td>
<td>1.027</td>
<td>76.8</td>
<td>1.028</td>
</tr>
<tr>
<td>3</td>
<td>34.5</td>
<td>1.029</td>
<td>72.1</td>
<td>1.027</td>
</tr>
<tr>
<td>3</td>
<td>58.5</td>
<td>1.029</td>
<td>54.7</td>
<td>1.022</td>
</tr>
<tr>
<td>3</td>
<td>82.5</td>
<td>1.028</td>
<td>50.0</td>
<td>1.017</td>
</tr>
<tr>
<td>Culture with 175 mg L(^{-1}) of Go-Ferm® in Stage 1 and 175 mg L(^{-1}) of DAP in Stages 2 and 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.5</td>
<td>1.070</td>
<td>67.6</td>
<td>1.070</td>
</tr>
<tr>
<td>2</td>
<td>17</td>
<td>1.030</td>
<td>78.8</td>
<td>1.030</td>
</tr>
<tr>
<td>2</td>
<td>18</td>
<td>1.033</td>
<td>60.7</td>
<td>1.033</td>
</tr>
<tr>
<td>3</td>
<td>42</td>
<td>1.025</td>
<td>61.0</td>
<td>1.023</td>
</tr>
<tr>
<td>3</td>
<td>62</td>
<td>1.014</td>
<td>61.6</td>
<td>1.014</td>
</tr>
</tbody>
</table>

In the first buildup attempt using *S. cerevisiae* EC 1118, when the yeast entered Stage 3, they were unable to consume the sugars as the specific gravity remained unchanged in the control/no treatment and bentonite treated buildups. As the time progressed during Stage 3 from 34.5 hours to 84.5 hours, there was an 20% approximate decline in cell viability for both control/no treatment and bentonite treated buildup. The pectinase treated buildup showed a decrease in specific gravity, yeast consuming sugar, as well as a steady decline in viability. Due to the lack of change in specific gravity and rapid cell death, new *liqueur de tirage* cultures were built up along with the appropriate
nutrients to help yeast through the acclimation and proliferation stages. The second part of Table 3.3 shows the specific gravity and cell viability of all treatments with the addition of 175 mg L\(^{-1}\) of Go-Ferm® in Stage 1 and 175 mg L\(^{-1}\) of DAP in Stages 2 and 3. The initial impact of Go-Ferm® in Stage 1 and DAP in Stage 2 can be observed as yeast were in Stage 2 for 3.5 hours less than without the added nutrients. The overall time for the buildup was approximately 20 hours shorter than without nutrients and an appropriate decline in specific gravity was observed. The cell viability remained at around 60% prior to bottling for all treatments.

3.4.3. Chemical analysis of final sparkling wines.

The chemical compositions of all finished Riesling sparkling wine treatments are given in Table 3.3.

**Table 3.3:** Chemical composition of finished Riesling treatments. Values represent the average ± standard deviation of the mean of triplicates. Statistical methods used were analysis of variance (ANOVA) with mean separation by Fisher’s Least Significant Difference (LSD; p<0.05). In descending order, lowercase letters indicate statistical difference between treatments using Fisher’s LDS\(_{0.05}\).

<table>
<thead>
<tr>
<th></th>
<th>Control BW</th>
<th>Pectinase BW</th>
<th>Bentonite BW</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-) Bentonite in tirage</td>
<td>3.1 ± 0.1 b</td>
<td>3.8 ± 0.6 ab</td>
<td>4.0 ± 0.6 ab</td>
</tr>
<tr>
<td>(+) Bentonite in tirage</td>
<td>3.8 ± 0.6 ab</td>
<td>4.6 ± 0.4 a</td>
<td>4.5 ± 1.1 a</td>
</tr>
<tr>
<td>Pressure (atm)</td>
<td>4.0 ± 0.6 ab</td>
<td>4.6 ± 0.4 a</td>
<td>4.7 ± 0.0 a</td>
</tr>
<tr>
<td>pH</td>
<td>2.85 ± 0.01 bc</td>
<td>2.87 ± 0.02 ab</td>
<td>2.89 ± 0.01 a</td>
</tr>
<tr>
<td>Titratable Acidity (g L(^{-1}))</td>
<td>7.5 ± 0.3 ab</td>
<td>7.7 ± 0.3 a</td>
<td>7.1 ± 0.1 c</td>
</tr>
<tr>
<td>Ethanol (% v/v)</td>
<td>12.7 ± 0.0 a</td>
<td>12.8 ± 0.0 a</td>
<td>12.7 ± 0.0 a</td>
</tr>
<tr>
<td>Glucose + Fructose (g L(^{-1}))</td>
<td>0.3 ± 0.0 c</td>
<td>0.7 ± 0.5 b</td>
<td>0.3 ± 0.0 bc</td>
</tr>
<tr>
<td>Malic acid (g L(^{-1}))</td>
<td>1.1 ± 0.0 a</td>
<td>1.1 ± 0.0 ab</td>
<td>1.1 ± 0.0 a</td>
</tr>
</tbody>
</table>
All six treatments successfully underwent secondary fermentation in bottle. According to the *liqueur de tirage* protocol for secondary fermentation, approximately 24 g L\(^{-1}\) of sugar is added to the base wine prior to bottling for yeast to consume and most of this sugar was consumed by the yeast. Successful fermentation was observed by the decrease in residual sugar amongst treatments from 24 g L\(^{-1}\) at the start of secondary fermentation to the ending concentration between 0.3-1.5 g L\(^{-1}\) (Table 3.3). Due to severe gushing at the disgorging stage in production, some pressure was lost as values range from 3.1-4.7 atm as opposed to achieving the expected 6 atm. The pH, TA and malic acid dropped in the juice to finished wine across all treatments. The final ethanol (% v/v) ranged from 12.4-12.8%. Even with the added water to dilute the base wine, the final alcohol was above the desired amount in sparkling wine.

**3.4.4. Protein analysis of juice and wine.**

The protein profiles from the initial juice to the juice post-settling, post-racking and pre-inoculation, as well as from pre-ferment to post-fermented base wine, are shown on Figure 3.2. Each treatment was first analysed on its own gel at the various stages of treatment. Figures 3.2 (A, C, E) depict the profiles of control/no treatment, pectinase treated and bentonite treated juice; there is an increase in protein, around 30 kDa, after settling and racking in the control and pectinase treated juices. The bentonite treated juice, as shown on Figure 3.2E lanes 4-6, shows that the addition of 2.0 g L\(^{-1}\) of sodium bentonite stripped all grape proteins as visually no bands were detected. Figures 3.2 (B, D, F) depict the profiles of the treatments before and after primary fermentation. Both control/no and pectinase treatments show a decline in volume intensity of the protein profile where the bands are less intense after fermentation. The profile of the bentonite treatment remains unchanged after primary fermentation (Figure 3.2F lanes 1-6).
Figure 3.2: Protein profile over the stages of base wine production: initial pressed Riesling juice to after settling, racked and pre-inoculated juice and the profile over fermentation of the pre-inoculated juice to post-fermentation base wine. The pre-inoculated juice samples are the same in both sets of left and right gels to observe the change in protein over sparkling wine production. Lanes: (PMr) Protein Marker, (1) Rep 1, (2) Rep 2, (3) Rep 3, (4) Rep 1, (5) Rep 2, (6) Rep 3, (7) 25µg mL⁻¹ BSA standard.
**Figure 3.3**: (A) Protein concentration in initial pressed Riesling juice treatments and post-treatment and pre-inoculated juice. (B) Protein concentration in before and after primary fermentation of Riesling treatments determined using densitometry from SDS-PAGE gels. Values represent the average ± standard deviation of the mean of triplicates. Statistical methods used were analysis of variance (ANOVA) with mean separation by Fisher’s Least Significant Difference (LSD; p<0.05). In descending order, lowercase letters indicate statistical difference before and after primary fermentation stages using Fisher’s LDS_{0.05}.

Figure 3.3A shows a significant difference between the total protein concentration at initial pressed juice and pre-inoculated juice stages across all treatments. In Figure 3.3B, there is a significant
difference between pre-ferment and post-fermentation protein concentrations for the control/no treatment and pectinase treatment. For the bentonite treatment, there is no indication of any yeast protein release after the primary fermentation since there was no significant difference in protein concentration between the pre-ferment juice and post-fermentation base wine.

All the juice treatments were also compared side-by-side on the same gel after all juice processing was performed (Figure 3.4A) and after primary fermentation (Figure 3.4B). As observed in Figure 3.2 and Figure 3.3, the addition of 2.0 g L\(^{-1}\) sodium bentonite also showed a significantly reduced protein profile at the pre- (Figure 3.4A) and post- (Figure 3.4B) ferment stages and protein concentration (Figure 3.5) when compared to the control/no treatment and pectinase treatment.

When comparing the protein profile between treatments at the pre- and post-ferment stages, a similar profile is observed between control and pectinase treatment (Figure 3.4A/B lanes 1-6) and no visible profile is observed in bentonite treatment.

**Figure 3.4:** Protein profile of control/no treatment and bentonite treated at a given time point in Riesling sparkling wine production. Lanes: (PMr) Protein Marker, (1) Control rep 1, (2) Control rep 2, (3) Control rep 3, (4) Pectinase rep 1, (5) Pectinase rep 2, (6) Pectinase rep 2, (7) Bentonite rep 1, (8) Bentonite rep 2 and (9) Bentonite rep 3.
There was no significant difference in protein concentration between control and pectinase treatment before inoculation and after primary fermentation to base wine (Figure 3.5).

Figure 3.5: Protein concentration of Riesling treatments at a given time point before and after primary fermentation determined using densitometry from SDS-PAGE gels. Values represent the average ± standard deviation of the mean of triplicates. Statistical methods used were analysis of variance (ANOVA) with mean separation by Fisher’s Least Significant Difference (LSD; p<0.05). In descending order, lowercase letters indicate statistical difference in protein concentration between treatments at a given stage in primary fermentation of sparkling wine production using Fisher’s LDS0.05.

The protein profile over the course of three stages in secondary fermentation appears relatively unchanged over time (Figure 3.6). The protein bands observed in all treatments range from approximately 20-30 kDa (Figure 3.6). There was the presence of one band between 20-25 kDa that appeared in both bentonite treatments (+/- bentonite in tirage) from time zero in secondary fermentation to the profile in the finished sparkling wines (Figures 3.6E and F).
**Figure 3.6:** Protein profile over the course of three timepoints (time zero, lees aging midpoint and finished wine) in secondary fermentation for Riesling. Lanes: (PMr) Protein Marker, (1) Time zero rep 1, (2) Time zero rep 2, (3) Time zero rep 3, (4) Midpoint rep 1, (5) Midpoint rep 2, (6) Midpoint rep 3, (7) Final wine rep 1, (8) Final wine rep 2 and (9) Final wine rep 3.
The pectinase treatments (+/- bentonite in tirage) had the highest protein concentrations throughout the stages of secondary fermentation (Figure 3.7).

**Figure 3.7**: Protein concentration over the course of secondary fermentation in bottle of Riesling base wine to finished sparkling wine determined using densitometry from SDS-PAGE gels. Values represent the average ± standard deviation of the mean of triplicates. Statistical methods used were analysis of variance (ANOVA) with mean separation by Fisher’s Least Significant Difference (LSD; p<0.05). In descending order, lowercase letters indicate statistical difference between timepoints using Fisher’s LDS$_{0.05}$.

The control treatments (+/- bentonite in tirage) had the second highest protein concentrations throughout secondary fermentation where both treatments are very similar in profile and concentration throughout (Figure 3.7). The bentonite treatments (+/- bentonite in tirage) had the lowest amount of total protein throughout secondary fermentation and a slight increase in total protein was observed for both treatments in the finished sparkling wine (Figure 3.7). The bentonite treatments (+/- bentonite in tirage) were significantly lower in total protein concentration than the other treatments at all three timepoints in secondary fermentation (Figure 3.7).

Figure 3.8 depicts the comparison of protein profiles between treatments without and with the addition of 0.95 mL L$^{-1}$ of Inoclair 2 (sodium bentonite) in the tirage.
Figure 3.8: Protein profile of final Riesling sparkling wine comparing the addition of Inoclair 2 (IOC, France) in tirage. Lanes: (PMr) Protein Marker, (1) No Inoclair addition rep 1, (2) No Inoclair addition rep 2, (3) No Inoclair addition rep 3, (4) Inoclair addition rep 1, (5) Inoclair addition rep 2, (6) Inoclair addition rep 3, (7) 25µg mL⁻¹ BSA standard.
All six treatments (control, pectinase and bentonite treatments (+/- bentonite in tirage)) showed that there was no significant difference between final Riesling wines with or without the addition of bentonite at secondary fermentation.

**Figure 3.9:** Protein profile concentration of final Riesling wine treatments determined using densitometry from SDS-PAGE gels. Values represent the average ± standard deviation of the mean of triplicates. Statistical methods used were analysis of variance (ANOVA) with mean separation by Fisher’s Least Significant Difference (LSD; p<0.05). In descending order, lowercase letters indicate statistical difference between no Inoclar 2 addition and Inoclar 2/bentonite addition in tirage between treatments a using Fisher’s LDS$_{0.05}$. 

![Protein profile concentration of final Riesling wine treatments](image)
According to Table 3.4, both pectinase with bentonite treatments (+/- bentonite in tirage) were significantly lower in total protein compared to other treatments as determined by both protein determinations.

**Table 3.4:** Protein concentration in finished sparkling Riesling wines determined from BCA assay and densitometry from SDS-PAGE. Analysis was performed in triplicate and samples from each treatment were tested in duplicate (n=6) for both the BCA assay and densitometry from SDS-PAGE. Values represent the average ± standard deviation of the mean of triplicates. Statistical methods used were analysis of variance (ANOVA) with mean separation by Fisher’s Least Significant Difference (LSD; p<0.05). In descending order, lowercase letters indicate statistical difference in protein concentration between final sparkling wine treatments.

<table>
<thead>
<tr>
<th>Protein Concentration (µg mL⁻¹)</th>
<th>Control BW</th>
<th>Pectinase BW</th>
<th>Bentonite BW</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(-) Bentonite in tirage</td>
<td>(+) Bentonite in tirage</td>
<td>(-) Bentonite in tirage</td>
</tr>
<tr>
<td>BCA Assay</td>
<td>20.6 ± 2.3 a</td>
<td>20.9 ± 4.2 a</td>
<td>22.0 ± 5.0 a</td>
</tr>
<tr>
<td>Densitometry</td>
<td>62.0 ± 2.6 b</td>
<td>55.9 ± 6.0 b</td>
<td>107.2 ± 16.6 a</td>
</tr>
</tbody>
</table>

When comparing the protein concentrations from the BCA assay, there was no significant difference between control and pectinase treatments (+/- bentonite in tirage). The final protein concentration using densitometry showed a significant difference between control and pectinase treatments. Figure 3.10 is a visual comparison of final wine treatments on one gel where the higher protein load can be visually seen in the lanes where wine was fermented with pectinase treated juice (Figure 3.10, lanes 3 and 4) compared to the control (Figure 3.10, lanes 1 and 2). The bentonite treatments (+/- bentonite in tirage) only appear to have one prominent band at around 20kDa (Figure 3.10, lanes 5 and 6) while the remaining four treatments have prominent bands between 20-30kDa.
Figure 3.10: Protein profile of all Riesling sparkling wine treatments. Lanes: (PMr) Protein Marker, (1) Control BW + no additional treatment, (2) Control BW + Inoclar addition, (3) Pectinase BW + no additional treatment, (4) Pectinase BW + Inoclar addition, (5) Bentonite BW + no additional treatment, (6) Bentonite BW + Inoclar addition, (7) 25µg mL⁻¹ BSA standard.

3.4.5. Foaming analysis of final sparkling wines.

The time for dissipation of bubbles ranged from approximately 28-38 seconds (Figure 3.11A). Shorter times may have been observed due to the decrease in pressure from gushing and lost wine during the disgorging process. The bentonite treated juice treatments (+/- bentonite in tirage) had the longest times before the bubbles dissipated. Both bentonite treatments (+/- bentonite in tirage) also had one of the highest pressures (Table 3.3) and thus possibly losing the least amount of wine under pressure during disgorging. The control treatments (+/- bentonite in tirage) were higher in final volume than the remaining treatments (Figure 3.11B). The control treatments (+/- bentonite in tirage) also had the lowest final pressure than the other treatments (Table 3.2).
Figure 3.11: Foaming analysis of finished Riesling sparkling wine treatments. Values represent the average ± standard deviation of the mean of triplicate treatments. Statistical methods used were analysis of variance (ANOVA) with mean separation by Fisher’s Least Significant Difference (LSD: p<0.05). In descending order, lowercase letters indicate statistical difference between final sparkling wine treatments.
3.5. Discussion

3.5.1. Challenges in culture buildup for secondary fermentation

The initial tirage culture without the addition of 175 mg L\(^{-1}\) of Go-Ferm® in Stage 1 and 175 mg L\(^{-1}\) of DAP in Stages 2 and 3 did not successfully build up and reach an appropriate specific gravity (1.000-1.010) to enter secondary fermentation in bottle (Table 3.2). Once the amount of yeast increased and the nutrients were added at the appropriate stages, *S. cerevisiae* could carry through the acclimation and proliferation stages of *tirage* and successful ferment in bottle. This indicates that appropriate nutrients may be required in the *tirage* build up for base wines such as the Riesling used in this experiment. A study by Martí-Raga *et al.* (2015) found that factors such as yeast strain, fermentation temperature and nitrogen added for secondary fermentation impact the development and kinetics of secondary fermentation. The study determined nitrogen only impacts secondary fermentation kinetics when the concentration is below 30 mg N L\(^{-1}\) (Martí-Raga *et al.* 2015). It was suggested that the low-sugar and low-biomass production during secondary fermentation results in the lower nitrogen requirements when compared to requirements for primary fermentation. Primary fermentations require a minimum of 140-200 mg N L\(^{-1}\) (Arias-Gil *et al.* 2007). The Riesling treatments in primary fermentation had approximately 275 mg N L\(^{-1}\) with DAP addition. The finished base wines without any further additions had between 24 – 31 mg N L\(^{-1}\), which is bordering on what is required by the yeast. With the addition of 175 mg L\(^{-1}\) of DAP to the *tirage* and base wine, the total YANC would have been over the minimum 30 mg N L\(^{-1}\) concentration and thus not limiting yeast during acclimation and proliferation in the *tirage*.

Wine is a complex matrix where factors such as the ethanol concentration and pH in the base wine could have also limited yeast growth and caused additional stress on the *tirage* yeast, and thus appropriate nutrients were required during *tirage*. According to Borrull *et al.* (2014), ethanol is the
main component in base wine that affects yeast performance in secondary fermentation as it impacts its viability. The base wines of all three Riesling treatments were around 12% (v/v) ethanol due to a higher than desirable level of soluble solids in the initial pressed juice and fermenting the juice to dryness. Diluting the base wine was necessary to reach a desirable final ethanol concentration in the base wine and in the final wine. After a 1.12-fold dilution, the base wines remained around 11% (v/v) ethanol. Ethanol can be toxic to organisms (Bauer and Pretorius 2000). The S. cerevisiae cultures may have faced ethanol stress during the initial build up without the nutrients. Ethanol stress can result in a destruction of normal membrane structures and an increase membrane permeability (Bauer and Pretorius 2000). Although the TA was lower across treatments than the desired TA (10-12 g L\(^{-1}\)) in base wine for sparkling wine production, the pH was quite low and well below the desirable range of 3.0-3.3 pH. The TA in the final wines was around 7 g L\(^{-1}\) while the pH was around 2.85 amongst treatments (Table 3.3). During the build up for secondary fermentation, the acidic conditions may have impacted the yeast viability in the initial tirage without the added yeast micronutrient Go-Ferm® and nitrogen source from DAP (Table 3.2). An acidic environment such as what was found in the base wines can result in inhibition of growth of yeast cells, since weak acids can diffuse across the cell membrane when in an undissociated state. The intracellular pH forces the acids to dissociate and release H\(^+\) inside the cell when weak acids enter the cytosol (Mira et al. 2010). The release of H\(^+\) inhibits yeast metabolic functions as a decrease in intracellular pH is observed.

Since yeast experience stress from ethanol, acids, carbon dioxide and temperature, appropriate procedures during the acclimation and proliferation stages, including nutrient addition, are required for successful secondary fermentation in bottle. Go-Ferm® is a natural yeast rehydration nutrient consisting of multiple micronutrients to aid in fermentation. The use of Go-Ferm® has
been found to enhance fermentation rates, particularly in challenging environments such as low fermentation temperature and low pH (Threlfall and Morris 2009). The study by Threlfall and Morris (2009) found that the addition of 0.3 g L\(^{-1}\) and 0.6 g L\(^{-1}\) of Go-Ferm® increased micronutrients in the juice (magnesium and calcium) and the addition shortened the length of fermentation. Our results indicate that the addition of the micronutrient Go-Ferm® and the nitrogen addition from DAP to the low pH Riesling base wine allowed the yeast to overcome these stresses and build up a successful tirage.

### 3.5.2. Protein profile and concentration during sparkling wine production

The protein profile for all control and pectinase treatments (+/- bentonite in tirage) was similar throughout production with higher levels indicated in the pectinase treatments. The protein profile for the combined pectinase and 2.0 g L\(^{-1}\) bentonite treatments (+/- bentonite in tirage) were significantly lower when compared to the four other treatments, starting after the initial juice treatment and throughout production as the grape proteins were completely removed (Figure 3.2F). Figures 3.2 A and C show the protein profile of the control and pectinase treated juice at the initial juice stage (after pressing) and prior to inoculation for primary fermentation (after clarification and racking). There was an increase in protein concentration after 72 hours of clarification and settling (Figure 3.3A). From their protein profiles (Figures 3.2 A and C), a more prominent band appears around 30 kDa. Based on the literature, the proteins around 30 kDa are chitinases and Thaumatin-like proteins (TLPs) as their masses range from 20-30 kDa (Lira et al. 2014). Both groups of proteins also decrease in concentration with fermentation and this was observed in Figures 3.2 B and D. The increase in volume intensity of the 30 kDa band suggests that during settling and clarification of juice, proteins may be released or microbes indigenous to the grape may be increasing the protein concentration. However, the prominent band at 30 kDa is not as
intense after primary fermentation in both control/no treatment and pectinase treatment (Figure 3.4). The decrease in protein concentration after fermentation may be due to proteolytic activities and changing pH occurring in the wine leading to protein denaturation (Blasco et al. 2011). The bentonite treated juice showed no change in protein profile or concentration before and after primary fermentation (Figures 3.3A and B). The lack of protein bands present in the bentonite juice treatments and the decrease in protein in the other two after fermentation indicate that yeast cells used in primary fermentation have not yet undergone autolysis at the time the wines were racked off the yeast lees, and therefore, the protein profiles have not increased or been altered.

Furthermore, it has been reported that a decrease in protein concentration after fermentation may be attributed to the larger size of ethanol molecules compared to water allowing for better separation of bentonite layers and increasing their interaction capacity with proteins (Lira et al. 2014).

The presence of yeast proteins in the time zero wine after tirage addition from the dual pectinase/bentonite treatment appear to alter the profile during at the initial stage of secondary fermentation. The protein profiles and concentrations of all six Riesling treatments during secondary fermentation (time zero, lees aging midpoint and finished wine) are shown in Figures 3.6 and 3.7, respectively. Both pectinase with bentonite treatments (+/- bentonite in tirage) show a visual change in protein profile at the start of secondary fermentation with a band appearing between 20-30 kDa and remaining through to the finished wine (Figures 3.6 E and F). Similar to those treatments, the remaining four also appear to have a similar protein profile over the course of secondary fermentation where no new bands are observed but there appears to be an increase in protein concentration over time (Figures 3.6 A-D). This may be due to an increase in volume intensity of the band around 20-30 kDa. The bands are present at time zero in the wines from the
bentonite treatments and then persist over time, indicating that proteins are being released into the wine at the start of secondary fermentation. It is likely these proteins originate from the dead yeast that were part of the tirage build up. Since the dead yeast were not separated from the live yeast in the tirage upon addition of tirage to the individual sparkling wine bottles, autolysed yeast released protein that can be detected at time zero of the secondary ferment. Furthermore, the addition of Inoclar 2 (liquid sodium bentonite) in the tirage did not appear to significantly alter the protein profiles of the treatments (Figure 3.8). Very faint bands also appear around 70 kDa in the final wines (Figure 3.8). According to the literature, this band may be from grape invertase present in the final wine (Blasco et al. 2011). In this experiment, the addition of Inoclar 2 did not impose a large reduction of proteins in the final wine (Figure 3.9). The amount of Inoclar 2 (0.95 mL L⁻¹) does not appear to be sufficient to remove yeast proteins after secondary fermentation. It is used to assist with yeast settling but does not appear to remove protein.

Although the protein concentrations determined using the BCA assay are lower than those obtained from densitometry, the trend is similar. The composition of proteins in the juice and wine may be affecting the differences in protein concentration results between the BCA assay and densitometry. All control and pectinase treatments (+/- bentonite in tirage) are significantly higher in protein than the two bentonite treatments removed of grape proteins. The addition of bentonite at a concentration of 2.0 g L⁻¹ significantly alters the protein profiles and decreases the protein concentrations.

3.5.3. Foaming properties in finished sparkling wine

The finished Riesling wines heavily gushed upon disgorging and dosage addition. The final pressures can be found in Table 3.3 and values were not in the desired range for finished sparkling wine (5-6 atm). Over-foaming during fermentation can lead to a loss of foaming properties in the
The gushing observed at disgorging not only reduced the pressure of the finished wine but possibly also decreased the concentration of foam-active compounds as wine was lost. Although the dosage consisted of finished wine, the wine itself was subject to filtration and SO₂ addition to preserve the finished product which negatively impacts foam. Higher ethanol concentrations also negatively impact the foaming properties in sparkling wine (Cilindre et al. 2010; Blasco et al. 2011). According to Figures 3.11 A and B, the 2.0 g L⁻¹ bentonite treatments (+/- bentonite in tirage) had the longest time before the bubbles dissipated and the lowest final wine volumes. The lower wine volume could indicate that more foam was produced upon pouring, which resulted in the longest time for the foam to persist. It is also possible that the dual pectinase/bentonite treatments (+/- bentonite in tirage) lost the least amount of wine due to gushing. Furthermore, both pectinase/bentonite (+/- bentonite in tirage) treatments had the highest pressures in the finished wine (Table 3.3). Inversely, the control and pectinase treatments (+/- bentonite in tirage) had the highest final wine volume and the shortest observed dissipation of bubbles (Figure 3.11 A and B). Regardless of pressure loss, the foaming results indicate that the protein present in secondary fermentation (yeast proteins) positively contribute to the foaming properties in Riesling sparkling wine. The lack of grape proteins present in the 2.0 g L⁻¹ bentonite treatments (+/- bentonite in tirage) demonstrate that the yeast proteins are foam forming. Yeast mannoproteins have been shown to aid in foam formation and stabilization by having the ability to interact with the foam (bubble) walls from the hydrophilic glycans in mannoproteins (Vincenzi et al. 2014). Although they did not increase the length of dissipation time, the grape proteins in Riesling may potentially be foam stabilizers. The removal of grape invertase has been shown to decrease foam quality as grape invertase has beneficial surface properties (Dambrouck et al. 2005).
3.6. Conclusion

Ethanol and acid stress are two main limitations for yeast growth during *tirage* build up for secondary fermentation. Appropriate addition and timing of nutrients is required during the acclimation and proliferation stages of build up to ensure a viable culture and secondary fermentation in bottle. The use of pectinase on juice before primary fermentation did not change the grape protein profile. The addition of 2.0 g L$^{-1}$ of sodium bentonite completely stripped the grape proteins from the bentonite treated juice. The masses of proteins present in the final wine ranged from 20-30 kDa. The protein profile among all treatments remained similar and a small increase in protein was observed over the course of secondary fermentation. The addition of sodium bentonite (Inoclar 2) in *tirage* did not remove proteins in the final wine. Furthermore, the amount of Inoclar 2 was not sufficient for flocculation of yeast cells after fermentation in riddling prior to disgorging. The loss of wine and pressure from disgorging and *dosage* impacted the foaming properties in the finished wine. Yeast proteins contribute to the foamability in Riesling sparkling wine. Appropriate oenological practices including a longer riddling period may positively impact the suitability of Riesling for quality sparkling wine production.
3.7. Literature Cited


Chapter 4

4. The role of grape varieties and grape clones on sparkling wine quality

4.1. Abstract

Aims

The objective of this study was to understand the impact of different grape varietals, clones and soil types on sparkling wine quality. The aim was also to understand how clones may differ in initial grape protein profiles, to observe the changing profile over the course of sparkling wine production and to ascertain the impact of that protein profile on final sparkling wine quality.

Methods and Results

Four Chardonnay clones, two Pinot noir clones, Chardonnay musqué on two different soils and three Riesling clones on different soils were selected for this study. Grapes from Chardonnay, Pinot noir, Chardonnay musqué and Riesling clones were harvested and processed. All Chardonnay, Pinot noir and Chardonnay musqué treatments were treated with a pectinase enzyme and split into three replicates. The Riesling treatments were split with the use of two different pectinase enzymes and fermented in four replicates due to larger harvest volumes. Treatments underwent settling and racking prior to primary fermentation. All treatments were inoculated with commercial wine yeast, Saccharomyces cerevisiae EC 1118. After the completion of primary fermentation, base wines were subjected to racking, blending, cold stabilization and filtering. A tirage culture for each treatment was made and all varietals/clones were inoculated with the same yeast for secondary fermentation in bottle. Successful fermentation in bottle was completed and wines were subjected to chemical and protein analyses from the initial juice to the midpoint of lees aging in sparkling wine production. The wines were not subject to foaming analysis as the final
sparkling wines are not included in this specific study. A decrease in protein concentration was observed in all clone, soil and enzyme treatments over the course of sparkling wine production excluding Riesling treatments. At the lees aging midpoint, Chardonnay clone 548 had the highest protein concentration when compared to the other evaluated Chardonnay clones. The Chardonnay musqué from the clay soil had a higher protein concentration compared to the sandy soil treatment at the lees aging midpoint. The protein concentrations were not significantly different between both evaluated Pinot noir clones at the lees aging midpoint. Riesling clone 239 on SO4 rootstock grown on sandy soil and treated with the KS enzyme had the highest protein concentration amongst Riesling treatments.

Conclusions

Clones from the same varietal possess varying chemical and protein compositions. The four Chardonnay clones and two Chardonnay musqué treatments had the desired initial juice chemical composition which carried through into the wine and can be used for quality sparkling wine production. Final protein concentration varies between varietals and may impact the foaming properties and quality of finished sparkling wines. Based on the protein concentrations at the lees aging midpoint, Riesling had the highest concentrations and may have the most positive impact on foaming properties. The soil composition appeared to impact protein concentration; however this appears to be specific to grape varietal and applied oenological practice as a higher level was observed in the Chardonnay musqué clay soil treatment. In contrast, the 239 Riesling clone grown on SO4 rootstock in sandy soil and treated with KS enzyme in the juice had higher protein concentration determined from densitometry.
Significance and Impact

This study provides an initial database and insight into grape varietals and clones that may be suitable for quality sparkling wine in the Niagara Peninsula of Ontario, Canada. These results demonstrate what impacts soil composition and various oenological practices, such as the use of pectinases for settling, may have on the final protein composition. This project will provide grape growers and winemakers information on which clones may be suitable for the higher tier sparkling products, for lower tier sparkling wine or for table wine production.

Keywords: sparkling wine, winemaking, varietal, Chardonnay, Pinot noir, Chardonnay musqué, Riesling, soil, clones, pectinase, protein, yeast, S. cerevisiae, SDS-PAGE, BCA assay

4.2. Introduction

Oenological practices during sparkling wine production such as addition of bentonite and length of lees aging impact the finished wine quality. However, optimizing sparkling wine quality starts in the vineyard by applying appropriate viticultural practices and using the best varieties and clones suited to soil type. Grapes used for sparkling wine production typically have lower pH, high titratable acidity and lower soluble solids when compared to grapes used for table wine production (Jones et al. 2014). The pH, soluble solids and titratable acidity are used to determine when grapes are harvested and impact wine quality (Jones et al. 2014). Factors such as grape variety, clone, yield, berry composition, fruit maturity, disease, soil and climate can impact the quality of grapes harvested for production (Pozo-Bayón et al. 2009; Pelsy 2010).

Along with vineyard location and yeast autolysis, grape variety plays a major role in defining quality sparkling wine (de La Presa-Owens et al. 1998). Chardonnay and Pinot noir are two of the three varietals used for traditional Champagne production and are commonly used for sparkling
wine production in the Niagara Peninsula. Chardonnay is defined as providing “elegance and finesse” while Pinot noir provides “body” in traditional sparkling wines (Jackson 2008). Other varietals including Chardonnay musqué, a Muscat-type flavour and clone of Chardonnay, as well as Riesling, are also currently used in Niagara Peninsula sparkling wines. Distinct aroma compounds can be associated with a specific varietal or clone of a varietal, and the desired aromas in the final wine may also influence the grapes used for sparkling wine production. Furthermore, the maturation rate and potential foaming properties can be driving factors in clonal or varietal choice for sparkling wine production (Jones et al. 2014). Chardonnay has been found to have the highest foam forming properties (Blasco et al. 2011). Pinot noir has been found to have the greatest foam height (Marchal et al. 2001).

The range of clonal diversity in a grape variety depends on the age of the variety, as the older the variety, the longer the exposure to environmental stress that may result in mutations establishing a clone (Pelsy 2010). Clones of a variety can show divergent genotypes that can lead to expression of a different phenotype (Pelsy 2010). The differences between clones possibly exist as a response to the environment, random mutations or presence of viruses resulting in epigenetic modifications (Kaeppler et al. 2000).

A study by Wolpert et al. (1994) evaluated the performance of six Chardonnay clones in California for still wine production. After comparison of the clones from two vineyard sites over three years, researchers concluded that there were clonal differences in yield, berries/cluster and cluster size (Wolpert et al. 1994). Another study by Fidelibus et al. (2006), evaluated the yield and fruit composition of six Chardonnay clones in California used for still wine production. Clonal differences were also observed in berry weight, cluster weight, berries per cluster as well as chemically with varying pH, TA and soluble solids amongst clones (Fidelibus et al. 2006). These
results indicate that chemical composition and grape maturity differences between clones can affect sparkling wine quality. There has been little published work on optimizing clone choice for sparkling wine production.

The presence of pests also influences fruit composition, protein profile and foaming properties of sparkling wines. Heavy rainfall and other environmental factors may result in fungal presence in the vineyard. The presence of *Botrytis cinerea* on clusters leads to a release of proteases which degrade grape proteins in the initial juice (Esteruelas et al. 2014). The resulting degradation has been shown to reduce foaming properties in sparkling wine (Marchal et al. 2001). Soil composition can also impact vine vigor and berry weight because of vine water status (Jones et al. 2014). Evaluating traditional sparkling wine varietals and different clones will provide insight into understanding optimal vineyard practices for quality sparkling wine production.

### 4.3. Materials and Methods

#### 4.3.1. Experimental Design.

**Figure 4.1**: Breakdown of grape varietals and clones used in sparkling clonal trial.
4.3.2. Yeast.

Primary and secondary fermentations were completed using commercial yeast *Saccharomyces cerevisiae* Lalvin EC 1118 supplied by Lallemand Inc. (Montreal, QC, Canada).

4.3.3. Grape juice.

To investigate the role of grape varietal on sparkling wine quality, various Chardonnay, Pinot noir, Chardonnay musqué and Riesling clones were harvested from the Niagara Peninsula and whole bunch pressed to 1.5 bar in the Pilot Winery at Brock University (St. Catharines, ON, Canada). SO₂ was added to the pressed juice at a concentration of 50 ppm total sulfur dioxide and clonal/soil treatments were separated into 20 L plastic carboys. The Chardonnay, Pinot noir and Chardonnay musqué treatments were separated into triplicates while the Riesling treatments were separated into four replicates due to larger juice volumes. The four Chardonnay clones evaluated were 95, 548, 127 and 128. The two Pinot noir clones used in this experiment were clones 459 and 386. The two Chardonnay musqué treatments were one from sandy soil and one from clay soil. The Riesling clones evaluated were 239 on SO4 rootstock on sandy soil, 49 on SO4 rootstock on sandy soil, and 239 on SO4 rootstock on clay soil. Each variety and clone received an enzyme addition for juice clarification with a Scottzyme KS enzyme, a blended enzyme product consisting of predominately pectinase (Scott Laboratories Ltd, CAN). Riesling clones were further separated, where half of the Riesling treatments clone 239 on SO4 rootstock sandy soil and clone 49 on SO4 rootstock sandy soil were treated with the Scottzyme KS enzyme and the other half with LAFAZYM®, a pectinase used for juice clarification (Laffort, Bordeaux, France). All juice treatments were then allowed to settle at 4°C for 24 hours. After settling, the juice was racked and inoculated.
4.3.4. Chemical analyses of juice and wine.

Soluble solids of the initial juices were determined with an ABBE bench top refractometer (Model 10450, American Optical, Buffalo, NY, USA). Reducing sugar concentration was measured per manufacturer’s instruction from a commercial enzyme assay kit (K-FRUGL) (Megazyme International Ireland Ltd; Wicklow, IRE). Juice and wine pH was determined with a Corning pH meter (model 455) with a calibration of pH 4.0, pH 7.0 and pH 10.0. Titratable acidity (TA g L$^{-1}$ tartaric acid) was determined by titration with 0.1 N NaOH to an endpoint of pH 8.2 (Zoecklein et al. 1996). Yeast assimilable nitrogen content (YANC) is the sum of primary amino nitrogen (PAN) and ammonia nitrogen. PAN and ammonia in the juice and base wine was measured using K-PANOPA chemical assay and K-AMIAR enzymatic kit, respectively (Megazyme International Ireland Ltd; Wicklow, IRE). Malic acid was measured per manufacturer’s instructions from a commercial enzyme assay kit (K-LMAL) (Megazyme International Ireland Ltd; Wicklow, IRE). Free and total sulfur dioxide (SO$_2$) measurements were performed using aeration-oxidation method on wine samples. Ethanol analysis on wine samples was measured via gas chromatography (Agilent, CA, USA) using an Agilent 6890 model coupled with a flame ionization detector (GC-FID), DB Wax column (30 m x 0.25 mm x 0.25 μm), split/ split-less injector and Chemstation software. The pressure of sparkling wines after secondary fermentation was determined using an aphrometer (Afriso Eurogauge Ltd, West Sussex, UK).

4.3.5. Protein analyses of juice and wine.

Protein analyses of samples were carried out from the start to finish of sparkling wine production. Initial protein concentration results were determined with the Bradford assay per manufacturer’s instructions (Sigma-Aldrich, St. Louis, MO, USA). Due to pectin interference with the Bradford assay and reducing sugar interference with the BCA assay, samples were subjected to protein
precipitation using an acetone precipitation method (Wessel and Flügge 1984). Once precipitation was complete, the precipitated proteins were dissolved in water. The BCA assay was performed on the precipitated proteins from the finished wine samples following the manufacturer’s instructions (Fisher Scientific, Hampton, NH, USA). Bovine serum albumin (BSA) was used for the calibration curve. Due to the reducing sugar interferences with the BCA assay, even after precipitating the proteins, predominately in juice, all samples from every major stage in winemaking were subjected to protein determination using densitometry using SDS-PAGE. For SDS-PAGE analysis, proteins were separated with 12% polyacrylamide gels pH 8.8, 5% stacking gels pH 6.8 and run at 1 hour and 40 minutes at 100V. Gel images were scanned and densitometry was performed using the Bio-Rad Image Lab software (Bio-Rad, Mississauga, ON, CAN).

Total protein in juice or wine was calculated based on the densitometry signal from the SDS-PAGE gels. A 20 µL volume of juice or wine was separated by SDS-PAGE and stained with a SYPRO Ruby stain. Instructions for the SYPRO Ruby stain were followed from manufacturer’s guidelines (Bio-Rad, Mississauga, ON, CAN). A protein ladder (10µL) was also run on each gel in order to act as the reference for protein quantification (Unstained Precision Plus Protein™; Bio-Rad, Mississauga, ON, CAN). The total protein signal from the densitometry signal in each lane from the SDS-PAGE gels was quantified with reference to the densitometry signal for the 50 kDa band (750 ng) when 10 µL of ladder was run on the gel. It was shown that the protein quantification signal from densitometry was linear over a wide range of protein signals (see Appendix I graph A) and that the 50 kDa band in the ladder was a good reference band to use for normalization to control for gel to gel variation in staining (see Appendix I graphs A and B).

The total protein amount (ng) in 20 µL of juice or wine was quantified by comparing the total densitometry signal from each lane to that found for the 50 kDa band of 10 µL of the protein ladder.
on that same gel. The 50 kDa band represents 750 ng of protein when 10 µL of the ladder is run out on the gel (Unstained Precision Plus Protein™; Bio-Rad, Mississauga, ON, CAN).

Protein concentration in the juice or wine sample (ng/µL) =

(Total protein signal / 50 kDa signal) x (750 ng / 20 µL volume of juice or wine extract)

4.3.6. Sample collection and timepoints.

Juice and wine samples for chemical and protein analysis were collected at every stage of winemaking. The soluble solids, pH, TA, SO₂, Bradford assay and YAN analyses were performed on fresh juice or wine samples. The remaining samples for analyses of residual sugar (glucose and fructose), ethanol, malic acid, BCA, SDS-PAGE and ethanol were collected, centrifuged at 10 000 rpm for 5 min to remove grape particulate and yeast cells (Sorvall RC5C Plus, rotor model SLA-3000; Newtown, CT, USA). The samples were then filtered using a 0.45 µm filter pad (Millipore, Sigma-Aldrich) and aliquoted into 1-250 mL plastic Nalgene container (Thermo-Fisher, Waltham, MA, USA) and 5-1.5 mL Eppendorf tubes (Eppendorf Canada, Mississauga, ON, CAN). After centrifugation and filtration, samples were frozen at -20ºC until analyzed.

4.3.7. Primary fermentation and winemaking.

Primary fermentation of juice to base wine was carried out in 20 L plastic containers. The juice was inoculated at an amount of 0.3 g L⁻¹ and prepared per manufacturer’s instructions (Lallemand Inc, CAN). Go-Ferm® rehydration nutrient (Danstar Ferment A.G., CH) was added to the rehydration preparation at an amount of 0.3 g L⁻¹. Nitrogen supplement, diammonium phosphate (DAP) was added to target 275 mg N L⁻¹, as insufficient YAN concentration was found across all varietals and clones in the juice. The plastic fermenters were moved to a fermentation chamber at Brock University that was maintained at 18ºC for the first 48 hours and then moved to a 16 ºC for the remainder of fermentation. Carboys were monitored daily for temperature and sugar
consumption. During fermentation, sugar consumption was monitored using a °Brix hydrometer and temperature was monitored using a thermometer labelled in degrees Celsius. Primary fermentation was considered complete when the specific gravity was less than 1.000 and showed no further decline for three days. To confirm that fermentations had gone to dryness (less than 5 g L\(^{-1}\) reducing sugar), wine samples were drawn from the fermenters and underwent sugar determination using a FOSS wine scan (WineScan™; Hillerød, DK) as well as the sugar enzymatic kit (K-FRUGL; Megazyme International Ireland Ltd; Wicklow, IRE). Potassium metabisulfite was then added to the base wines a concentration of 40 ppm SO\(_2\), blanketed with CO\(_2\) and moved to 4ºC. The base wines then were racked off their lees, replicates were blended for each treatment and returned to cold storage at -2ºC. The free SO\(_2\) levels in the base wines were monitored weekly and to maintain a level of 20 ppm SO\(_2\), additions were made accordingly. To help cold stabilize the base wines, potassium bitartrate (Vines to Vintages, Vineland, ON, CAN) was added at 4 g L\(^{-1}\) and stirred thoroughly. The base wines were placed at -4 ºC to cold stabilize and were then racked. The base wines were then filtered using a 0.8 μm Seitz KS80 filter pad (Pall Food & Beverage, NY, USA) to have a turbidity reading below 1.5 NTU. Riesling base wines were diluted 1.12-fold with Milli-RiOs water (RiOS-16; Millipore, Etobicoke, ON, CAN) to decrease the ethanol to average 11.0% ethanol. With the dilution, sucrose additions for secondary fermentation in bottle allowed for approximately 1.5-2% increase in ethanol in finished sparkling wines.

4.3.8. Yeast inoculation procedure for tirage and secondary fermentations.

All varietals and treatments for secondary fermentation were inoculated with *Saccharomyces cerevisiae* Lalvin EC 1118 (Montreal, QC, CAN). The tirage build up consisted of three stages. In Stage 1, the commercial *S. cerevisiae* EC 1118 yeast was rehydrated according to manufacturer’s instructions for secondary fermentation at 0.1 g L\(^{-1}\). Cultures from Stage 1 were then combined
with equal parts base wine and liqueur (base wine with 500 g L$^{-1}$ added sugar) to enter Stage 2. These cultures were fermented at the lab bench at 22ºC and were subjected to regular mixing, cell counts with a hemocytometer and monitoring specific gravity to observe sugar consumption. Once cultures had reached a specific gravity of 1.030, Stage 3 commenced with the further addition of wine, liqueur and water. Specific gravity and cell counts continued throughout this stage. The tirage cultures were ready to be added to the base wine to start secondary fermentation in bottle when the specific gravity attained a range of 1.000-1.010.

Due to the amount of treatments, bottling was scheduled for two days and thus tirage cultures were built up per their treatment’s bottling date. All Riesling treatments appeared to have a healthy starter culture and were subsequently bottled. According to the Riesling trial in Chapter 3, the initial liqueur de tirage buildup did not appear to consume sugars as the specific gravity remained unchanged and viable cell counts decreased over time. To prevent a similar issue with liqueur de tirage buildup, remaining treatments to be bottled (Chardonnay, Pinot noir and Chardonnay musqué treatments) received an increasing yeast amount of 0.15 g L$^{-1}$, addition of 175 mg L$^{-1}$ of Go-Ferm® in Stage 1, and DAP was added to the wine at an amount of 175 mg L$^{-1}$ in Stages 2 and 3.

To undergo secondary fermentation, residual sugar was analyzed and base wines received a sucrose addition for a total amount of approximately 24 g L$^{-1}$ residual sugar for yeast to consume. DAP was also added to the base wine used in secondary fermentation to help the yeast ferment at a concentration of 175 g L$^{-1}$. Bottling for secondary fermentation took place in the Pilot Winery at Brock University. Each tirage culture was based on a 60 L wine volume. According to this, the volume of tirage culture for a 750 mL ferment was calculated to be 22.5 mL. Approximately 727.5 mL of base wine from each treatment was first measured out in a 1000 mL plastic graduated
cylinder and poured using a funnel into the bottle. Then the tirage culture was added using the automatic pippetter (Eppendorf, Mississauga, ON, CAN). The bottle was then crown capped, labelled and placed on its side in the cage. Bottled wines were then transported to Fielding Estate Winery (Beamsville, ON, CAN) and stored at approximately 13 ºC for secondary fermentation and lees aging of 9 months. During secondary fermentation, sparkling wine samples were collected in triplicate for each treatment at time zero (the start of secondary fermentation) and half way through lees aging (midpoint) at 5 months. The samples collected at this timepoint were subjected to chemical analyses. Furthermore, at the midpoint, wines underwent pressure analysis of the bottles using an aphrometer (Afriso Eurogauge Ltd, West Sussex, UK) to determine if sugar consumption was occurring. The sparkling wines to be used for chemical analyses at the midpoint were centrifuged at 10 000 rpm for 5 minutes to remove yeast cells (Sorvall RC5C Plus, rotor model SLA-3000; Newtown, CT, USA) and then filtered using a 0.45 µm filter pad (Millipore). The scope of this project concludes with chemical and protein analyses of the sparkling wines at the midpoint of lees aging.

4.3.9. Statistical analysis.

Differences between variables were determined by the XLSTAT statistical software package released by Addinsoft (Version 7.1; Paris, France). Statistical methods were analysis of variance (ANOVA) with mean separation by Fisher’s Least Significant Difference (LSD; p<0.05).
4.4. Results

4.4.1. Chardonnay: Chemical analyses, fermentation kinetics and protein analyses.

The chemical compositions of all four Chardonnay clones at the pre-inoculated juice stage, post-ferment base wine and at the lees aging midpoint are given in Table 4.1. The initial soluble solids vary by approximately 1.5 ºBrix but are between 18.3 and 19.7 ºBrix. The pH and titratable acidity range from 3.02-3.04 and 10.9-11.7 respectively. Clone 95 is significantly lower in malic acid at the pre-inoculated juice stage. The consumption of soluble solids during primary fermentation is depicted in Figure 4.2.
Table 4.1: Chemical composition of Chardonnay clonal treatments on pre-inoculated juice, post-ferment base wine and at the lees aging midpoint. Values represent the average ± standard deviation of the mean of triplicates. Statistical methods used were analysis of variance (ANOVA) with mean separation by Fisher’s Least Significant Difference (LSD; p<0.05). In descending order, lowercase letters indicate statistical difference between treatments at a given timepoint using Fisher’s LDS_{0.05}.

<table>
<thead>
<tr>
<th>Pre-inoculated juice</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clones</strong></td>
<td>95</td>
<td>548</td>
<td>127</td>
<td>128</td>
</tr>
<tr>
<td>Soluble Solids (°Brix)</td>
<td>19.7 ± 0.0</td>
<td>19.6 ± 0.0</td>
<td>18.3 ± 0.0</td>
<td>18.6 ± 0.1</td>
</tr>
<tr>
<td>Glucose + Fructose (g L^{-1})</td>
<td>213 ± 4</td>
<td>184 ± 3</td>
<td>195 ±2</td>
<td>202 ± 0</td>
</tr>
<tr>
<td>pH</td>
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<td>3.02 ± 0.01</td>
<td>3.02 ± 0.00</td>
<td>3.04 ± 0.01</td>
</tr>
<tr>
<td>Titratable Acidity (g L^{-1})</td>
<td>10.9 ± 0.1</td>
<td>11.7 ± 0.1</td>
<td>11.3 ± 0.1</td>
<td>11.1 ± 0.1</td>
</tr>
<tr>
<td>Amino Nitrogen (mg N L^{-1})</td>
<td>93 ± 3</td>
<td>104 ± 0</td>
<td>83 ± 5</td>
<td>86 ± 1</td>
</tr>
<tr>
<td>Ammonia Nitrogen (mg N L^{-1})</td>
<td>71 ± 5</td>
<td>93 ± 2</td>
<td>82 ± 5</td>
<td>86 ± 1</td>
</tr>
<tr>
<td>Malic acid (g L^{-1})</td>
<td>4.5 ± 0.1</td>
<td>5.0 ± 0.1</td>
<td>4.9 ± 0.3</td>
<td>4.9 ± 0.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Post-ferment base wine</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>2.94 ± 0.03</td>
<td>2.94 ± 0.00</td>
<td>2.97 ± 0.00</td>
<td>3.00 ± 0.00</td>
</tr>
<tr>
<td>Titratable Acidity (g L^{-1})</td>
<td>11.9 ± 0.0</td>
<td>12.6 ± 0.1</td>
<td>12.2 ± 0.2</td>
<td>12.2 ± 0.1</td>
</tr>
<tr>
<td>Ethanol (% v/v)</td>
<td>11.5 ± 0.2</td>
<td>11.2 ± 0.2</td>
<td>10.8 ± 0.0</td>
<td>11.0 ± 0.1</td>
</tr>
<tr>
<td>Glucose + Fructose (g L^{-1})</td>
<td>2.9 ± 0.8</td>
<td>2.2 ± 0.6</td>
<td>0.8 ± 0.0</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>Malic acid (g L^{-1})</td>
<td>3.8 ± 0.1</td>
<td>4.3 ± 0.1</td>
<td>4.3 ± 0.0</td>
<td>4.4 ± 0.0</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Lees aging midpoint in sparkling wine</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pressure (atm)</strong></td>
<td>5.4 ± 0.1</td>
<td>5.6 ± 0.2</td>
<td>6.0 ± 0.1</td>
<td>5.7 ± 0.0</td>
</tr>
<tr>
<td>pH</td>
<td>2.90 ± 0.00</td>
<td>2.92 ± 0.00</td>
<td>2.95 ± 0.00</td>
<td>2.90 ± 0.00</td>
</tr>
<tr>
<td>Titratable Acidity (g L^{-1})</td>
<td>11.6 ± 0.0</td>
<td>12.2 ± 0.0</td>
<td>11.9 ± 0.0</td>
<td>11.7 ± 0.0</td>
</tr>
<tr>
<td>Ethanol (% v/v)</td>
<td>13.1 ± 0.0</td>
<td>13.2 ± 0.0</td>
<td>12.5 ± 0.0</td>
<td>12.8 ± 0.0</td>
</tr>
<tr>
<td>Glucose + Fructose (g L^{-1})</td>
<td>0.3 ± 0.0</td>
<td>0.4 ± 0.0</td>
<td>0.4 ± 0.0</td>
<td>0.3 ± 0.0</td>
</tr>
<tr>
<td>Malic acid (g L^{-1})</td>
<td>3.1 ± 0.0</td>
<td>3.6 ± 0.0</td>
<td>3.2 ± 0.1</td>
<td>3.6 ± 0.1</td>
</tr>
</tbody>
</table>
Figure 4.2: Primary fermentation kinetics of all Chardonnay juice clones inoculated with S. cerevisiae EC 1118. Fermentations were performed in triplicates. Soluble solid values represent the average ± standard deviation of the mean from triplicate fermentations.

Similar fermentation kinetics were observed between the four clones where all completed fermentation around day 11. As expected due to lower initial soluble solids, the ethanol concentration (% v/v) after fermentation was lower in clones 127 and 128 (Table 4.1). The residual sugar in the base wines were also significantly lower in clones 127 and 128. The chemical composition of the clones at the lees aging midpoint indicate that all treatments successfully underwent secondary fermentation in bottle as between 5.4-6.0 atm of pressure was observed (Table 4.1). The alcohol at the midpoint ranged from 12.5-13.2% (v/v) and residual sugar from 0.3-0.4 g L⁻¹. The pH also dropped at the lees aging midpoint to 2.90-2.95 amongst clones.

Figures 4.3 and 4.4 show the differences between Chardonnay clones in protein profile and protein concentration respectively. Figure 4.3 tracks the protein profile from the pre-inoculated juice through to the protein in the wine at the lees aging midpoint.
Figure 4.3: Protein profile of Chardonnay clones (95, 548, 127 and 128) at three winemaking timepoints (A) pre-inoculated juice, (B) base wine and (C) lees aging midpoint. Lanes: (PMr) Protein Marker, (1) clone rep 1, (2) clone rep 2, (3) clone rep 3, (4) clone rep 1, (5) clone rep 2, (6) clone rep 3.
A faint protein band can be observed around 70 kDa across the four clones at all sampling timepoints (Figure 4.3). More prominent protein bands are observed between 20-25 kDa and appear less intense throughout production (Figure 4.3).

**Figure 4.4**: Protein profile concentration over the course of sparkling wine production of Chardonnay clonal treatments and determined using densitometry from SDS-PAGE gels. Values represent the average ± standard deviation of the mean of triplicates. Statistical methods used were analysis of variance (ANOVA) with mean separation by Fisher’s Least Significant Difference (LSD; p<0.05). In descending order, lowercase letters indicate statistical difference between treatments at a given timepoint using Fisher’s LDS0.05.

According to Figure 4.3, there is no significant difference between Chardonnay clones at the pre-inoculated juice stage. At the lees aging midpoint, clones 95 and 548 are significantly higher in protein concentration than clones 127 and 128 (Figure 4.4). A comparison of protein concentration at the lees aging midpoint determined using the BCA assay and densitometry from SDS-PAGE can be found in Table 4.2.
Table 4.2: Protein concentration in Chardonnay clones at lees aging midpoint determined from BCA assay and densitometry from SDS-PAGE. Values represent the average ± standard deviation of the mean of triplicates analyzed in duplicate (n=6). Statistical methods used were analysis of variance (ANOVA) with mean separation by Fisher’s Least Significant Difference (LSD; p<0.05). In descending order, lowercase letters indicate statistical difference in protein concentration between treatments.

<table>
<thead>
<tr>
<th>Clones</th>
<th>Protein (µg mL⁻¹)</th>
<th>BCA Assay</th>
<th>Densitometry</th>
</tr>
</thead>
<tbody>
<tr>
<td>95</td>
<td>11.3 ± 0.8 b</td>
<td>40.7 ± 2.2 a</td>
<td></td>
</tr>
<tr>
<td>548</td>
<td>16.8 ± 1.5 a</td>
<td>48.8 ± 6.3 a</td>
<td></td>
</tr>
<tr>
<td>127</td>
<td>15.6 ± 2.3 a</td>
<td>23.9 ± 3.8 b</td>
<td></td>
</tr>
<tr>
<td>128</td>
<td>15.2 ± 3.0 a</td>
<td>21.2 ± 4.8 b</td>
<td></td>
</tr>
</tbody>
</table>

According to the BCA assay, the protein concentration ranges from 11.3-16.8 µg mL⁻¹ and clone 95 is significantly lower than the others. According to densitometry, the protein concentration ranges from 21.2-48.8 µg mL⁻¹ and clones 95 and clones 548 are significantly higher than clones 127 and 128.
4.4.2. Chardonnay musqué: Chemical analyses, fermentation kinetics and protein analyses.

The chemical compositions of the two Chardonnay musqué soil treatments at the pre-inoculated juice stage and at the lees aging midpoint are given in Table 4.3.

**Table 4.3:** Chemical composition of Chardonnay musqué soil treatments on pre-inoculated juice, post-ferment base wine and at the lees aging midpoint. Values represent the average ± standard deviation of the mean of triplicates. Statistical methods used were analysis of variance (ANOVA) with mean separation by Fisher’s Least Significant Difference (LSD; p<0.05). In descending order, lowercase letters indicate statistical difference between treatments at a given timepoint using Fisher’s LDS_{0.05}.

<table>
<thead>
<tr>
<th></th>
<th>Pre-inoculated juice</th>
<th>Post-ferment base wine</th>
<th>Lees aging midpoint in sparkling wine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>809 Sandy Soil</td>
<td>809 Clay Soil</td>
<td></td>
</tr>
<tr>
<td>Soluble Solids (°Brix)</td>
<td>19.5 ± 0.0 b</td>
<td>19.8 ± 0.1 a</td>
<td>Soluble Solids (°Brix)</td>
</tr>
<tr>
<td>Glucose + Fructose (g L⁻¹)</td>
<td>226 ± 5 a</td>
<td>220 ± 10 a</td>
<td>Glucose + Fructose (g L⁻¹)</td>
</tr>
<tr>
<td>pH</td>
<td>3.19 ± 0.01 a</td>
<td>3.20 ± 0.00 a</td>
<td>pH</td>
</tr>
<tr>
<td>Titratable Acidity (g L⁻¹)</td>
<td>9.4 ± 0.0 a</td>
<td>8.7 ± 0.0 b</td>
<td>Titratable Acidity (g L⁻¹)</td>
</tr>
<tr>
<td>Amino Nitrogen (mg N L⁻¹)</td>
<td>114 ± 0 b</td>
<td>136 ± 1 a</td>
<td>Amino Nitrogen (mg N L⁻¹)</td>
</tr>
<tr>
<td>Ammonia Nitrogen (mg N L⁻¹)</td>
<td>49 ± 0 b</td>
<td>65 ± 1 a</td>
<td>Ammonia Nitrogen (mg N L⁻¹)</td>
</tr>
<tr>
<td>Malic acid (g L⁻¹)</td>
<td>4.4 ± 0.0 a</td>
<td>3.7 ± 0.1 a</td>
<td>Malic acid (g L⁻¹)</td>
</tr>
<tr>
<td></td>
<td>11.4 ± 0.1 a</td>
<td>10.0 ± 0.1 b</td>
<td></td>
</tr>
<tr>
<td>Ethanol (% v/v)</td>
<td>11.4 ± 0.1 a</td>
<td>10.0 ± 0.1 b</td>
<td>Ethanol (% v/v)</td>
</tr>
<tr>
<td>Glucose + Fructose (g L⁻¹)</td>
<td>0.9 ± 0.2 b</td>
<td>2.4 ± 0.9 a</td>
<td>Glucose + Fructose (g L⁻¹)</td>
</tr>
<tr>
<td>Malic acid (g L⁻¹)</td>
<td>3.8 ± 0.2 a</td>
<td>3.4 ± 0.3 b</td>
<td>Malic acid (g L⁻¹)</td>
</tr>
<tr>
<td></td>
<td>5.1 ± 0.1 b</td>
<td>6.0 ± 0.0 a</td>
<td></td>
</tr>
<tr>
<td>Pressure (atm)</td>
<td>3.11 ± 0.01 a</td>
<td>3.10 ± 0.00 a</td>
<td>Pressure (atm)</td>
</tr>
<tr>
<td>pH</td>
<td>3.08 ± 0.03 a</td>
<td>3.08 ± 0.03 a</td>
<td>pH</td>
</tr>
</tbody>
</table>

Although the compositions are similar, there are significant differences between soil treatments in soluble solids, amino nitrogen, ammonia nitrogen and malic acid at the juice stage. The consumption of soluble solids during primary fermentation is depicted in Figure 4.5.
Figure 4.5: Primary fermentation kinetics of all Chardonnay musqué juice treatments inoculated with *S. cerevisiae* EC 1118. Fermentations were performed in triplicate. Soluble solid values represent the average ± standard deviation of the mean from triplicate fermentations. Similar fermentation kinetics were observed between the treatments where both reached completion around day 11. After primary fermentation, there are significant differences between soil treatments in all chemical parameters except pH. The chemical composition of the soil treatments at the lees aging midpoint indicate that all treatments successfully underwent secondary fermentation in bottle as between 5.1-6.0 atm of pressure was observed (Table 4.3). The alcohol at the midpoint ranged from 13.0-13.6% (v/v) and residual sugar from 1.2-1.9 g L\(^{-1}\). There was no significant difference in pH between soil treatments at the lees aging midpoint.

Figures 4.6 and 4.7 show the differences between Chardonnay musqué soil treatments in protein profile and protein concentration, respectively. Figure 4.6 depicts the protein profile from the pre-inoculated juice through to the protein in the wine at the lees aging midpoint.
**Figure 4.6:** Protein profile of Chardonnay musqué (clone 809) soil treatments at three winemaking timepoints (A) pre-inoculated juice, (B) base wine and (C) lees aging midpoint. Lanes: (PMr) Protein Marker, (1) 809 Sandy Soil (SS) rep 1, (2) 809 SS rep 2, (3) 809 SS rep 3, (4) 809 Clay Soil (CS) rep 1, (5) 809 CS rep 2, (6) 809 CS rep 3.
A faint protein band can be observed around 70 kDa in both sandy and clay soil samples and decrease in intensity over the course of production (Figure 4.6). More prominent protein bands are observed at around 20-30 kDa and appear less intense throughout sparkling wine production (Figure 4.6).

**Figure 4.7:** Protein concentration over the course of sparkling wine production of Chardonnay musqué soil treatments and determined using densitometry from SDS-PAGE gels. Values represent the average ± standard deviation of the mean of triplicates. Statistical methods used were analysis of variance (ANOVA) with mean separation by Fisher’s Least Significant Difference (LSD; p<0.05). In descending order, lowercase letters indicate statistical difference between treatments at a given timepoint using Fisher’s LDS$_{0.05}$. 
A comparison of protein concentration at the lees aging midpoint determined using the BCA assay and densitometry from SDS-PAGE can be found in Table 4.4.

Table 4.4: Protein concentration in Chardonnay musqué soil treatments at lees aging midpoint determined from BCA assay and densitometry from SDS-PAGE. Values represent the average ± standard deviation of the mean of triplicates analyzed in duplicate (n=6). Statistical methods used were analysis of variance (ANOVA) with mean separation by Fisher’s Least Significant Difference (LSD; p<0.05). In descending order, lowercase letters indicate statistical difference in protein concentration between treatments.

<table>
<thead>
<tr>
<th>Soil Treatment</th>
<th>Protein (µg mL⁻¹)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>BCA Assay</td>
<td>Densitometry</td>
</tr>
<tr>
<td>809 Sandy Soil</td>
<td>14.5 ± 1.4 a</td>
<td>33.1 ± 3.8 b</td>
<td></td>
</tr>
<tr>
<td>809 Clay Soil</td>
<td>20.7 ± 5.8 a</td>
<td>51.0 ± 1.4 a</td>
<td></td>
</tr>
</tbody>
</table>

The BCA assay showed no significant differences in protein concentration between Chardonnay musqué sandy and clay soil treatments where the protein concentration ranged from 14.5-20.7 µg mL⁻¹. According to densitometry, the wine obtained from grapes grown in the clay soil is significantly higher in protein concentration (51.0 µg mL⁻¹) than compared to its sandy soil counterpart (33.1 µg mL⁻¹). At each stage in production, the clay soil treatment maintains a significantly higher level of protein concentration (Figure 4.6).
4.4.3. Pinot noir: Chemical analyses, fermentation kinetics and protein analyses.

The chemical compositions of the two Pinot noir clones (459 and 386) at the pre-inoculated juice stage, post-fermentation base wine and at the lees aging midpoint are given in Table 4.5.

**Tables 4.5:** Chemical composition of Pinot noir clone treatments on pre-inoculated juice, post-fermentation base wine and at the lees aging midpoint. Values represent the average ± standard deviation of the mean of triplicates. Statistical methods used were analysis of variance (ANOVA) with mean separation by Fisher’s Least Significant Difference (LSD; p<0.05). In descending order, lowercase letters indicate statistical difference between treatments at a given timepoint using Fisher’s LDS0.05.

<table>
<thead>
<tr>
<th></th>
<th>Clones</th>
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<th>386</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pre-inoculated juice</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soluble Solids (°Brix)</td>
<td></td>
<td>18.7 ± 0.1 b</td>
<td>19.2 ± 0.0 a</td>
</tr>
<tr>
<td>Glucose + Fructose (g L⁻¹)</td>
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<td>162 ± 1 b</td>
<td>198 ± 9 a</td>
</tr>
<tr>
<td>pH</td>
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<td>3.03 ± 0.01 a</td>
<td>3.04 ± 0.01 a</td>
</tr>
<tr>
<td>Titratable Acidity (g L⁻¹)</td>
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<td>10.4 ± 0.1 a</td>
<td>9.4 ± 0.1 b</td>
</tr>
<tr>
<td>Amino Nitrogen (mg N L⁻¹)</td>
<td></td>
<td>61 ± 1 a</td>
<td>53 ± 0 b</td>
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<tr>
<td>Ammonia Nitrogen (mg N L⁻¹)</td>
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<td>40 ± 0 a</td>
<td>30 ± 0 b</td>
</tr>
<tr>
<td>Malic acid (g L⁻¹)</td>
<td></td>
<td>3.8 ± 0.0 a</td>
<td>3.4 ± 0.1 b</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Post-ferment base wine</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>2.93 ± 0.01 a</td>
<td>2.93 ± 0.00 a</td>
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</tr>
<tr>
<td>Titratable Acidity (g L⁻¹)</td>
<td>11.3 ± 0.0 a</td>
<td>10.5 ± 0.1 b</td>
<td></td>
</tr>
<tr>
<td>Ethanol (% v/v)</td>
<td>11.2 ± 0.2 a</td>
<td>11.3 ± 0.1 a</td>
<td></td>
</tr>
<tr>
<td>Glucose + Fructose (g L⁻¹)</td>
<td>0.7 ± 0.2 b</td>
<td>3.2 ± 0.8 a</td>
<td></td>
</tr>
<tr>
<td>Malic acid (g L⁻¹)</td>
<td>3.6 ± 0.0 a</td>
<td>3.0 ± 0.1 b</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Lees aging midpoint in sparkling wine</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Pressure (atm)</td>
<td>5.6 ± 0.1 a</td>
<td>5.7 ± 0.1 a</td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>2.90 ± 0.01 a</td>
<td>2.92 ± 0.00 a</td>
<td></td>
</tr>
<tr>
<td>Titratable Acidity (g L⁻¹)</td>
<td>9.2 ± 0.0 b</td>
<td>9.9 ± 0.0 a</td>
<td></td>
</tr>
<tr>
<td>Ethanol (% v/v)</td>
<td>12.9 ± 0.0 b</td>
<td>13.1 ± 0.0 a</td>
<td></td>
</tr>
<tr>
<td>Glucose + Fructose (g L⁻¹)</td>
<td>0.3 ± 0.0 b</td>
<td>2.4 ± 0.2 a</td>
<td></td>
</tr>
<tr>
<td>Malic acid (g L⁻¹)</td>
<td>3.0 ± 0.0 a</td>
<td>2.6 ± 0.0 b</td>
<td></td>
</tr>
</tbody>
</table>

There are significant differences between clones in soluble solids, glucose/fructose, titratable acidity, amino nitrogen, ammonia nitrogen and malic acid at the juice stage. The consumption of soluble solids during primary fermentation is depicted in Figure 4.8.
Figure 4.8: Primary fermentation kinetics of all Pinot noir juice clones inoculated with \textit{S. cerevisiae} EC 1118. Fermentations were performed in triplicate. Soluble solid values represent the average ± standard deviation of the mean from triplicate fermentations.

Similar fermentation kinetics were observed between the treatments, where both reached completion around day 14. No significant differences in ethanol concentration (\% v/v) and pH were observed after primary fermentation of the two clones. The chemical composition of the clones at the lees aging midpoint indicate that all treatments successfully underwent secondary fermentation in bottle as between 5.6-5.7 atm of pressure was observed (Table 4.5). The alcohol at the midpoint ranged from 12.9-13.1\% (v/v) and residual sugar from 0.3-2.4 g L$^{-1}$.

Figures 4.9 and 4.10 show the protein profile and protein concentration from clones 459 and 386 over the course of sparkling wine production. Figure 4.9 depicts the protein profile from the pre-inoculated juice through to the protein in the wine at the lees aging midpoint.
Figure 4.9: Protein profile of Pinot noir clones at three winemaking timepoints (A) pre-inoculated juice, (B) post-ferment base wine and (C) lees aging midpoint. Lanes: (PMr) Protein Marker, (1) 459 rep 1, (2) 459 rep 2, (3) 459 rep 3, (4) 386 rep 1, (5) 386 rep 2, (6) 386 rep 3.
A faint protein band can be observed around 70 kDa in both clone treatments and decrease in intensity over the course of production (Figure 4.9). More prominent protein bands are observed at around 20-30 kDa and appear less intense throughout production (Figure 4.9). According to Figure 4.9, there are no significant differences between Pinot noir clones 459 and 386 at the three sampled timepoints.

![Figure 4.10: Protein concentration over the course of sparkling wine production of Pinot noir clone treatments and determined using densitometry from SDS-PAGE gels. Values represent the average ± standard deviation of the mean of triplicates. Statistical methods used were analysis of variance (ANOVA) with mean separation by Fisher’s Least Significant Difference (LSD; p<0.05). In descending order, lowercase letters indicate statistical difference between treatments at a given timepoint using Fisher’s LDS_{0.05}.](image-url)
A comparison of protein concentration at the lees aging midpoint determined using the BCA assay and densitometry from SDS-PAGE can be found in Table 4.6.

**Table 4.6:** Protein concentration in Pinot noir clonal treatments at lees aging midpoint determined from BCA assay and densitometry from SDS-PAGE. Values represent the average ± standard deviation of the mean of triplicates analyzed in duplicates (n=6). Statistical methods used were analysis of variance (ANOVA) with mean separation by Fisher’s Least Significant Difference (LSD; p<0.05). In descending order, lowercase letters indicate statistical difference in protein concentration between treatments.

<table>
<thead>
<tr>
<th>Clone</th>
<th>Protein (µg mL⁻¹)</th>
<th>BCA Assay</th>
<th>Densitometry</th>
</tr>
</thead>
<tbody>
<tr>
<td>459</td>
<td>11.5 ± 4.8 a</td>
<td>29.5 ± 6.3 a</td>
<td></td>
</tr>
<tr>
<td>386</td>
<td>10.1 ± 1.2 a</td>
<td>22.2 ± 5.0 a</td>
<td></td>
</tr>
</tbody>
</table>

Both the BCA assay and densitometry show no significant differences in protein concentration between clones. The protein concentration ranges from 10.1-11.5 µg mL⁻¹ and 22.2-29.5 µg mL⁻¹ when determined using the BCA assay and densitometry, respectively.

**4.4.4. Riesling: Chemical analyses, fermentation kinetics and protein analyses.**

The chemical compositions of all Riesling treatments at the pre-inoculated juice stage, after primary fermentation to base wine and at the lees aging midpoint are given in Table 4.7. Although the range in chemical composition is small, there are differences between clones and enzyme treatments at the pre-inoculated juice stage. Amino nitrogen, ammonia nitrogen and pH are slightly higher in clone 239/SO4 for both sandy and clay soil treatments (Table 4.7). The initial soluble solids vary from 19.8-20.7 °Brix. The consumption of soluble solids during primary fermentation is depicted in Figure 4.11.
Table 4.7: Chemical composition of Riesling treatments on pre-inoculated juice, post-fermentation base wine and at the lees aging midpoint. Analyses were performed in four replicates at the juice stage and base wine stage and in triplicate at lees aging where values represent the average ± standard deviation of the mean of triplicates. Statistical methods used were analysis of variance (ANOVA) with mean separation by Fisher’s Least Significant Difference (LSD; p<0.05). In descending order, lowercase letters indicate statistical difference between treatments at a given timepoint using Fisher’s LSD_{0.05}.

<table>
<thead>
<tr>
<th></th>
<th>239/SO4 Sandy Soil (KS)</th>
<th>239/SO4 Sandy Soil (LAFAZYM®)</th>
<th>49/SO4 Sandy Soil (KS)</th>
<th>49/SO4 Sandy Soil (LAFAZYM®)</th>
<th>239/SO4 Clay Soil (KS)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pre-inoculated juice</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soluble Solids (°Brix)</td>
<td>19.8 ± 0.0 d</td>
<td>20.5 ± 0.1 b</td>
<td>20.3 ± 0.0 c</td>
<td>20.3 ± 0.0 c</td>
<td>20.7 ± 0.0 a</td>
</tr>
<tr>
<td>Glucose + Fructose (g L(^{-1}))</td>
<td>209 ± 2 bc</td>
<td>234 ± 8 a</td>
<td>206 ± 0 bc</td>
<td>209 ± 0 c</td>
<td>212 ± 0 b</td>
</tr>
<tr>
<td>pH</td>
<td>3.01 ± 0.01 a</td>
<td>3.01 ± 0.03 a</td>
<td>2.94 ± 0.02 b</td>
<td>2.96 ± 0.01 b</td>
<td>3.01 ± 0.01 b</td>
</tr>
<tr>
<td>Titratable Acidity (g L(^{-1}))</td>
<td>7.1 ± 0.0 c</td>
<td>7.5 ± 0.0 a</td>
<td>7.2 ± 0.0 b</td>
<td>7.4 ± 0.1 a</td>
<td>7.2 ± 0.1 bc</td>
</tr>
<tr>
<td>Amino Nitrogen (mg N L(^{-1}))</td>
<td>61 ± 1 a</td>
<td>55 ± 0 b</td>
<td>49 ± 0 c</td>
<td>49 ± 0 c</td>
<td>47 ± 3 c</td>
</tr>
<tr>
<td>Ammonia Nitrogen (mg N L(^{-1}))</td>
<td>39 ± 1 a</td>
<td>25 ± 1 b</td>
<td>24 ± 0 c</td>
<td>24 ± 0 c</td>
<td>26 ± 1 b</td>
</tr>
<tr>
<td>Malic acid (g L(^{-1}))</td>
<td>1.8 ± 0.0 c</td>
<td>2.3 ± 0.0 a</td>
<td>1.8 ± 0.0 c</td>
<td>1.9 ± 0.1 b</td>
<td>1.5 ± 0.0 d</td>
</tr>
<tr>
<td><strong>Post-ferment base wine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>2.94 ± 0.01 a</td>
<td>2.93 ± 0.01 a</td>
<td>2.93 ± 0.01 a</td>
<td>2.94 ± 0.01 a</td>
<td>2.89 ± 0.0 b</td>
</tr>
<tr>
<td>Titratable Acidity (g L(^{-1}))</td>
<td>8.2 ± 0.0 b</td>
<td>8.4 ± 0.1 a</td>
<td>8.4 ± 0.1 a</td>
<td>8.4 ± 0.0 a</td>
<td>8.5 ± 0.1 a</td>
</tr>
<tr>
<td>Ethanol (% v/v)</td>
<td>12.2 ± 0.1 c</td>
<td>12.9 ± 0.2 b</td>
<td>12.4 ± 0.1 c</td>
<td>12.5 ± 0.3 c</td>
<td>13.3 ± 0.1 a</td>
</tr>
<tr>
<td>Glucose + Fructose (g L(^{-1}))</td>
<td>0.1 ± 0.1 b</td>
<td>0.3 ± 0.0 ab</td>
<td>0.3 ± 0.1 ab</td>
<td>0.3 ± 0.1 ab</td>
<td>0.3 ± 0.1 a</td>
</tr>
<tr>
<td>Malic acid (g L(^{-1}))</td>
<td>1.6 ± 0.0 b</td>
<td>1.9 ± 0.1 a</td>
<td>1.5 ± 0.0 b</td>
<td>1.6 ± 0.0 b</td>
<td>1.4 ± 0.1 c</td>
</tr>
<tr>
<td><strong>Lees aging midpoint in sparkling wine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pressure (atm)</td>
<td>5.1 ± 1.1 b</td>
<td>5.7 ± 1.1 b</td>
<td>4.9 ± 0.4 b</td>
<td>7.1 ± 0.0 a</td>
<td>6.1 ± 0.4 ab</td>
</tr>
<tr>
<td>pH</td>
<td>2.95 ± 0.01 a</td>
<td>2.93 ± 0.00 a</td>
<td>2.93 ± 0.01 a</td>
<td>2.92 ± 0.00 ab</td>
<td>2.89 ± 0.01 b</td>
</tr>
<tr>
<td>Titratable Acidity (g L(^{-1}))</td>
<td>8.2 ± 0.0 b</td>
<td>8.5 ± 0.0 a</td>
<td>8.4 ± 0.0 a</td>
<td>8.5 ± 0.0 a</td>
<td>8.5 ± 0.0 a</td>
</tr>
<tr>
<td>Ethanol (% v/v)</td>
<td>12.7 ± 0.0 b</td>
<td>13.1 ± 0.0 ab</td>
<td>13.0 ± 0.0 ab</td>
<td>12.7 ± 0.0 b</td>
<td>13.4 ± 0.0 a</td>
</tr>
<tr>
<td>Glucose + Fructose (g L(^{-1}))</td>
<td>0.4 ± 0.0 c</td>
<td>2.3 ± 0.1 a</td>
<td>0.9 ± 0.0 b</td>
<td>2.5 ± 0.3 a</td>
<td>2.3 ± 0.0 a</td>
</tr>
<tr>
<td>Malic acid (g L(^{-1}))</td>
<td>1.2 ± 0.1 b</td>
<td>1.5 ± 0.0 a</td>
<td>1.1 ± 0.0 c</td>
<td>1.1 ± 0.0 c</td>
<td>1.0 ± 0.0 d</td>
</tr>
</tbody>
</table>
Figure 4.11: Primary fermentation kinetics of all Riesling juice treatments inoculated with \textit{S. cerevisiae} EC 1118. Fermentations were performed in four replicates. Soluble solid values represent the average ± standard deviation of the mean from four replicate fermentations.

Similar fermentation kinetics were observed among the five treatments with all reaching completion around day 10. All Riesling treatments have above 12% (v/v) ethanol after primary fermentation. The residual sugar was also around 0.3 g L\(^{-1}\) indicating that the primary fermentation had gone to dryness (<5 g L\(^{-1}\)). The chemical composition of the clones at the lees aging midpoint indicate that all treatments successfully underwent secondary fermentation in bottle, as between 4.9-7.1 atm of pressure was observed (Table 4.7). With the 1.12-fold dilution of the base wine, the alcohol at the midpoint ranged from 12.7-13.4% (v/v) and residual sugar from 0.3-2.5 g L\(^{-1}\). The pH at the lees aging midpoint ranged from 2.89-2.95 amongst clones.

Figures 4.12 and 4.13 show the protein profile and concentration of different Riesling treatments respectively. Figure 4.12 shows the protein profile from the pre-inoculated juice through to the protein in the wine at the lees aging midpoint.
Figure 4.12: Protein profile of Riesling clones and soil treatments at three winemaking timepoints: pre-inoculated juice, base wine and lees aging midpoint. Treatment codes are: Sandy soil with KS enzyme (SS-KS), Sandy soil with LAFAZYM® (SS-LAF) and Clay soil with KS enzyme (CS-KS). Lanes: (PMr) Protein Marker, (1) SS-KS rep 1, (2) SS-KS rep 2, (3) SS-KS rep 3, (4) SS-LAFAZYM® rep 1, (5) SS-LAFAZYM® rep 2, (6) SS-LAFAZYM® rep 3, (7) CS-KS rep 1, (8) CS-KS rep 2, (9) CS-KS rep 3.
At around 70 kDa, a faint protein band can be observed across all treatments that decreases in intensity over the course of production (Figure 4.12). More prominent protein bands are observed between 20-30 kDa and appear less intense throughout production (Figure 4.12). As shown in Figure 4.13, clone 239/SO4 Sandy soil and KS enzyme treatment have a significantly higher amount of total protein at all three sampling time points.

Figure 4.13: Protein concentration over the course of sparkling wine production of Riesling treatments and determined using densitometry from SDS-PAGE gels. Values represent the average ± standard deviation of the mean of triplicates. Statistical methods used were analysis of variance (ANOVA) with mean separation by Fisher’s Least Significant Difference (LSD; p<0.05). In descending order, lowercase letters indicate statistical difference between treatments at a given timepoint using Fisher’s LDS_{0.05}.
The comparison of protein concentration at the lees aging midpoint determined using the BCA assay and densitometry from SDS-PAGE can be found in Table 4.8.

Table 4.8: Protein concentration in Riesling treatments at lees aging midpoint determined from BCA assay and densitometry from SDS-PAGE. Values represent the average ± standard deviation of the mean of triplicates analyzed in duplicate (n=6). Statistical methods used were analysis of variance (ANOVA) with mean separation by Fisher’s Least Significant Difference (LSD; p<0.05). In descending order, lowercase letters indicate statistical difference in protein concentration between finished sparkling wine treatments.

<table>
<thead>
<tr>
<th>Clone/Soil/Enzyme Treatment</th>
<th>Protein (µg mL⁻¹)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BCA Assay</td>
<td>Densitometry</td>
</tr>
<tr>
<td>239/SO4 Sandy Soil – (KS)</td>
<td>19.5 ± 1.3 a</td>
<td>92.9 ± 2.6 a</td>
</tr>
<tr>
<td>239/SO4 Sandy Soil – (LAFAZYM®)</td>
<td>20.3 ± 6.2 a</td>
<td>61.9 ± 5.2 b</td>
</tr>
<tr>
<td>49/SO4 Sandy Soil – (KS)</td>
<td>24.8 ± 7.8 a</td>
<td>51.6 ± 4.6 cd</td>
</tr>
<tr>
<td>49/SO4 Sandy Soil – (LAFAZYM®)</td>
<td>20.9 ± 6.8 a</td>
<td>50.8 ± 3.0 d</td>
</tr>
<tr>
<td>239/SO4 Clay Soil – (KS)</td>
<td>24.0 ± 3.9 a</td>
<td>60.8 ± 8.6 bc</td>
</tr>
</tbody>
</table>

According to the BCA assay, the protein concentration ranges from 19.5-24.8 µg mL⁻¹ and there are no significant differences between treatments at the lees aging time point. According to densitometry, the protein concentration range from 50.8-92.9 µg mL⁻¹. The 239/SO4 sandy soil treatments are significantly higher in total protein compared to the 239/SO4 on clay soil when determined using densitometry. There is no significant difference between the LAFAZYM® treated 239/SO4 sandy soil and 49/SO4 sandy soil (Table 4.8).

4.5. Discussion

4.5.1. Chardonnay

Differences in chemical composition between Chardonnay clones were observed in both the juice and lees aging midpoint. The heavy rain conditions between harvest dates of clones 95/548 and clones 127/128 may have resulted in a dilution of soluble solids in the juice of clones 127 and 128. The pH and the TA values of all four clones remained within desirable parameters for quality.
sparkling wine production (Table 4.1). Sparkling wine typically has a low pH ranging from 2.9-3.2 and a titratable acidity of 10-12 g L\(^{-1}\) tartaric acid (Borrull et al. 2015). The ethanol concentration in all clones was high at the lees aging midpoint ranging from 12.5-13.2% (v/v). Ethanol negatively impacts the foaming properties in wine (Pozo-Bayón et al. 2009). Similar to that mentioned in the literature, the protein concentration decreased over the course of fermentation (Lira et al. 2013). According to Figure 4.3, all four Chardonnay clones had low intensity protein bands present around 70 kDa and more prominent bands around 20-30 kDa. A protein band appearing around 70 kDa may be invertase and bands between 20-30 kDa may be chitinases and TLPs (Marangon et al. 2014). The final protein concentration in Chardonnay clones according to the BCA assay ranged from 11.3 – 16.8 µg mL\(^{-1}\) and 21.2 - 40.7 µg mL\(^{-1}\) using densitometry from SDS-PAGE. According to Caliari et al. (2014), the protein in a finished Brazilian Chardonnay sparkling wine determined using the Bradford assay was 34.1 ± 2.3 mg L\(^{-1}\) (µg mL\(^{-1}\)). The protein concentration, determined using the Bradford assay, in a 2012 German Chardonnay sparkling wine was 20.9 mg L\(^{-1}\) (µg mL\(^{-1}\)) (Jaecckels et al. 2017). Although a different technique was used to determine the concentration, the protein concentration of the Chardonnay clones in this study appears to have a similar range as the other reported Chardonnay sparkling wines. The four Chardonnay clones at the aging midpoint had similar protein concentrations to those of the Brazilian and German chardonnays. These commercial wines may have been put onto market without much lees aging, as neither study mentioned the length of lees aging which can impact the final protein concentration. Increased lees aging has been found to increase protein concentrations as mannoproteins are released into the wine (Rowe et al. 2010).
4.5.2. Chardonnay musqué

The chemical compositions of the two Chardonnay musqué soil treatments are shown on Table 4.4. Both soil treatments are in the desirable pH range for sparkling wine (Borrull et al. 2015). The nitrogen (amino and ammonia) was significantly lower in the juice from the sandy soil treatment (Table 4.3). Environmental factors such as water stress, vine water status and soil composition may have resulted in lower nitrogen values (Meier et al. 2016). The ethanol concentration was 13.0% (v/v) in the sandy soil wine and 13.6% (v/v) in the clay soil wine at the lees aging midpoint. The increased ethanol may negatively impact the foaming properties in the final sparkling wine (Pozo-Bayón et al. 2009). There was a decrease in protein concentration over the course of production in both treatments, however the sandy soil Chardonnay musqué treatment had a significantly lower protein concentration throughout all evaluated stages of sparkling production (Figure 4.6). Both treatments showed a similar protein profile (Figure 4.6) throughout fermentation, where less intense bands appeared around 70 kDa (potentially invertase) and more prominent bands between 20-30 kDa (potentially chitinases and TLPs) (Marangon et al. 2014). Based on the higher protein concentration, the clay soil Chardonnay musqué treatment may be better suited for quality sparkling wine production. Future analysis into the foaming properties at the final sparkling wine stage to observe the role of proteins on these treatments would confirm this speculation. Furthermore, a study by Coelho et al. (2009) examined the impact of soil type on volatiles in sparkling wine and found that clay and clay-calcareous soils with positive drainage and water-holding capacities produced sparkling wines richer in volatiles when compared to the wines that were produced from vines planted on sandy soils.
4.5.3. Pinot noir

According to Table 4.5, both Pinot noir treatments (clones 459 and 386) were harvested at ideal maturity for sparkling wine production, as the pH and TA were suitable for quality sparkling wine production. In base wine, a pH ranging from 3.0 to 3.3 and a titratable acidity between 10-12 g L\(^{-1}\) tartaric acid is ideal (Ribéreau-Gayon et al. 2006). The ethanol concentration at the lees aging midpoint was at 12.9% (v/v) in clone 459 and 13.1% (v/v) in clone 386, higher than the maximum ideal value of 12.5% in finished sparkling wines. The soluble solids in the initial juice resulted in both base wines having an ethanol concentration around 11% (v/v), an ideal concentration at this stage in production. The large jump in ethanol concentration after secondary fermentation may be attributed to possibly a larger concentration of yeast cells inoculated in bottle when compared to the amount of yeast that may be bottled at a commercial winery. The resulting pressure however, after secondary fermentation, was between 5-6 atm for both clones. Clones 459 and 386 had similar protein profiles (Figure 4.9) throughout fermentation where a faint band appeared around 70 kDa (possibly invertase) and a more prominent band between 20-30 kDa (possibly chitinases or TLPs) (Marangon et al. 2014). According to Table 4.6, the protein concentration, at the lees aging midpoint, determined from the BCA assay ranged from 10.1 – 11.5 µg mL\(^{-1}\) and 22.2 – 29.5 µg mL\(^{-1}\) from densitometry. According to Marchal et al. (1997), the protein concentration of a Champagne produced from Pinot noir grapes was 15.0 mg L\(^{-1}\) (µg mL\(^{-1}\)) and was determined using the Bradford assay. The protein concentration of a Brazilian Pinot noir sparkling wine was 15.6 ± 1.5 mg L\(^{-1}\) (µg mL\(^{-1}\)) (Caliari et al. 2014). Although a different method was used to determine the concentration, the protein concentration of the Pinot noir clones in this study appear to be within a similar range as the other reported sparkling wines made from Pinot noir grapes. Neither study stated the length of lees aging time, which may have impacted the final protein concentration.
4.5.4. Riesling

The Riesling clones for this project were harvested approximately a month after the Chardonnay, Chardonnay musqué and Pinot noir clones. The delayed harvest date, due to the re-entry interval period after spraying, resulted in grapes that were further ripened as observed with a decrease in titratable acidity in the juice around 7 g L⁻¹ for all treatments and at a concentration more suitable for table wine production. The soluble solids were also higher than those of the other varietals because of the later harvest date which resulted in an ethanol concentration around 12% (v/v) in the base wine; thus all Riesling wines were diluted prior to secondary fermentation (Table 4.7). The pH in the initial juice and sparkling wine at the midpoint was between 2.9-3.0 and within the ideal range for sparkling wine production (Ribéreau-Gayon et al. 2006). The ethanol concentration at the midpoint ranged between 12.7 – 13.4 % (v/v) (Table 4.7). Even with the dilution of the base wine prior to secondary fermentation, the ethanol in all Riesling treatments is above the maximum ideal value of 12.5% (v/v) ethanol in sparkling wine. Furthermore, there were significant differences in pressure between treatments. Clone 49 on SO4 rootstock with KS pectinase addition had an average pressure of 4.9 atm while the LAFAZYM® pectinase treatment had an average pressure of 7.1 atm at the midpoint. The varying pressures and higher ethanol concentrations may have been due to hand bottling the wine for secondary fermentation as compared to using a bottling line in a commercial setting. Wine and yeast culture were separately out for each bottle, which may have resulted in more variability.

All five Riesling treatments had a low intensity protein band present around 70 kDa and more prominent bands between 20-30 kDa (Figure 4.12). The protein band appearing around 70 kDa may be invertase and bands between 20-30 kDa may be chitinases and TLPs (Marangon et al. 2014). The protein concentration decreased in all treatments before and after primary fermentation.
Interestingly, the protein concentration in all treatments then increased when that of base wine is compared to the wine at the lees aging midpoint. This increase in concentration is similar to what was observed over secondary fermentation in the Riesling treatments in Chapter 3. Furthermore, a significant difference in protein concentration was observed between the sandy soil clone 239 on SO4 rootstock treatments (KS and LAFAZYM® pectinase) over the course of production. However, there was no significant difference in protein concentration between the sandy soil clone 49 on SO4 rootstock treatments (KS and LAFAZYM® pectinase) over the course of production until the lees aging midpoint. These results suggest that enzyme behaviour may be different across commercial enzyme products which result in changes to juice composition amongst clones of the same varietal.

Enzymes are often used in winemaking to help clarify juice and/or wine (Armada et al. 2010). Commercial pectinase enzymes, typically from *Aspergillus niger*, have three different activities, including pectin esterase, pectin lyase and polygalacturonase activity (Armada et al. 2010). The Scottzyme KS enzyme contains these three activities as well as cellulase and protease between 1-5% (Scott Laboratories Ltd, CAN). Although protease is a listed ingredient in the Scottzyme KS enzyme, it did not appear to have any effect on the protein profile in its treated juice and resulting wine (Figure 4.12A-B). The lack of activity may be due to the protease having the smallest concentration of all the enzymes in the product, as well as the possible influence of a low pH environment (around pH 3.00) resulting in inactive protease. The LAFAZYM® product contains polygalacturonase and β-glucanase activity (Laffort, Bordeaux, France). The differences in enzyme composition may have impacted the chemical and protein profile between treatments. A study by Armada et al. (2010) also found that the use of different enzymes for must clarification may impact aroma compounds in the resulting wine. Finally, the impact of enzyme addition may
differ between wines due to the chemical composition of the grapes and downstream winemaking practices (Itu et al. 2011).

The final protein concentration values in all Riesling treatments were lower as determined by the BCA assay and not significantly different. The final protein concentration values when determined from densitometry were higher and showed significant differences between Riesling clones, enzyme treatments and soil composition (Table 4.8). Extensive work into normalizing the protein data by comparing samples to the 50kDa reference band was done in order to appropriately compare concentrations for multiple treatments across various gels (see Appendix I). The differences between methods may be due to the low protein concentrations from the BCA determination reaching the lower limit (10 µg mL$^{-1}$) of the assay and the gels showing higher protein profile intensities between treatments.

4.5.5. Varietal comparison and potential foaming properties

The nitrogen composition in the juice was highest in Chardonnay clones followed by Chardonnay musqué, Pinot noir and Riesling. All varietals were within an ideal sparkling wine pH range in both juice and wine. The ethanol concentrations were all higher than 12.5% (v/v) across all varietals, which may result in decreased foaming properties in all treatments (Pozo-Bayón et al. 2009). On average, Riesling treatments had the highest protein concentration at the lees aging midpoint followed by Chardonnay musqué, Pinot noir and Chardonnay. Bentonite was not used during production. The future foaming analysis will provide insight into the role of grape proteins on foaming properties and whether different clones have better foaming properties. Literature suggests that Chardonnay wines are the best foam formers while Pinot noir wines provide the best foam height (Marchal et al. 2001; Blasco et al. 2011).
4.6. Conclusion

Grape variety does influence the chemical and protein composition in juice and resulting wine. Factors such as fruit maturity are varietal specific and can influence the harvest date. The clones from each varietal evaluated (Chardonnay, Chardonnay musqué, Pinot noir and Riesling) varied in pH, TA and soluble solids. The pH at the lees aging midpoint was within the desired range for sparkling wine (2.9-3.3). The impact of soil composition on protein concentration may be varietal specific. All Riesling treatments had the highest protein concentration at the lees aging midpoint and the protein concentration at this timepoint may influence the foaming properties in the finished wine. This work gives an initial insight into grape varietals and clones that may be suitable for quality sparkling wine in the Niagara Peninsula. The results from this project provide grape growers and winemakers information about which clones may be suitable for the higher tier sparkling products, lower tier sparkling products or those suitable for table wines.
4.7. Literature Cited


Chapter 5

5. Discussion and Conclusions

Sparkling wine is a wine style suitable for cool climate regions, as grapes from a specific vintage may not reach full maturity resulting in low pH, high titratable acidity and lower soluble solids. Low pH, high TA and lower soluble solids are markers typically found in juice resulting in quality sparkling wine. Traditional sparkling wine production requires the primary fermentation of grape juice into base wine and secondary fermentation of base wine into sparkling wine in bottle. The major factor defining sparkling wine quality is the foaming properties that are observed formed once poured. Furthermore, vineyard conditions and practices, grape varietal and yeast strain impact the final sparkling wine quality (Jones et al. 2014).

Proteins, which are grape and yeast derived, impact the foaming properties and wine quality. Although proteins are present in small concentrations in juice and wine ranging from 10-500 mg L\(^{-1}\) of protein, they play a large role on foamability and foam stability (Pozo-Bayón et al. 2009; Marangon et al. 2014). In Chapter 2, the final protein concentrations, determined from densitometry, in the final Mariafeld Pinot noir sparkling wines ranged from 52.9 – 75.5 µg mL\(^{-1}\). In Chapter 3, the final protein concentrations from densitometry across all Riesling treatments ranged from 23.4 – 115.5 µg mL\(^{-1}\). In Chapter 4, the final protein concentrations from densitometry across all evaluated varietals and treatments ranged from 21.2 – 92.9 µg mL\(^{-1}\). The protein concentrations from all three studies were similar to wine protein concentrations found in the literature. Similar to the Pinot noir study, Salazar et al. (2010) found that increasing the amount of bentonite decreases the foamability and foam persistence. Thus, limiting the use or addition of bentonite during sparkling wine production maintains the protein profile and has a positive effect on foaming properties. In Chapter 2, when no bentonite was added to Pinot noir juice and base
wine, the time for bubbles to dissipate was the longest, averaging 10 minutes and the final protein concentration in the wine was the highest. When 1.0 g L\(^{-1}\) of bentonite was added to the Pinot noir juice, the final protein concentration was lower and the dissipation time was more than 50% shorter than the control. The addition of 0.95 mL L\(^{-1}\) bentonite in the tirage resulted in a significantly lower final protein concentration and the dissipation time was 20% shorter than the control. The addition of 1.0 g L\(^{-1}\) bentonite in the juice and 0.95 mL L\(^{-1}\) bentonite in the tirage resulted in the lowest amount of protein and the shortest dissipation time. These results agree with those in the literature and highlight the role of grape proteins as good foam stabilizers and yeast proteins as foam formers (Blasco et al. 2011). Contrary to these results, the Riesling treatments in Chapter 3 showed increased protein concentrations across treatments over the course of primary fermentation and that the use of bentonite in the tirage did not decrease protein concentrations.

To evaluate the role of grape proteins in sparkling wine, an addition of 2.0 g L\(^{-1}\) bentonite to Riesling juice removed grape proteins and was compared to a control/no bentonite treated Riesling juice and a pectinase treated juice. The bentonite did remove all the grape proteins as shown by the lack of a protein bands on the SDS-PAGE gels. No significant increase in protein from any yeast proteins was observed in the 2.0 g L\(^{-1}\) bentonite treatment after primary fermentation. Contrary to this, a decrease in protein was observed in the control/no treatment and pectinase treatment after primary fermentation. Vincenzi et al. (2010) found similar results, where electrophoretic analysis showed no new bands after primary fermentation, indicating little to no contribution of yeast proteins after primary fermentation. A protein band around 20 kDa appeared from time zero through to the finished sparkling wine in the 2.0 g L\(^{-1}\) bentonite treatments (+/-bentonite in tirage). The remaining four Riesling treatments also showed a slight increase in protein concentration over the course of secondary fermentation. This protein increase likely
occurred from dead yeast that were added during the *tirage* addition. During the *tirage*, there was a decrease in cell viability. Dead cells are not separated from live cells in the *tirage* addition. The dead cells may have undergone autolysis and released protein into the base wine at the start of secondary fermentation.

Gushing at the disgorging stage decreased the final pressure in all treatments and affected the foaming properties in the wine, as foam-active compounds may have been removed. Therefore, it is impossible to conclude how the protein profile from the various Riesling treatments impacted final foam quality due to the incomplete riddling to settle the yeast to the neck of the bottle for disgorging, resulting in the gushing activity. However, in the bentonite juice treatment to remove grape protein, the foaming analysis indicated that the proteins present during secondary fermentation (yeast proteins) positively affected the foamability. This is stated as the two 2.0 g L⁻¹ bentonite treatments had the longest foam dissipation time and no grape proteins present. The lack of grape proteins from these two 2.0 g L⁻¹ bentonite treatments (+/- bentonite in *tirage*) also show that the yeast proteins present in the final sparkling wine are foam forming/promote foamability. The over-foaming at disgorging may have resulted in a decrease in grape proteins from the other treatments as the initial foam across all treatments was not maintained for longer than 1 minute. The grape proteins in Riesling may potentially be foam stabilizers but due to the gushing issue, this could not be evaluated in this study.

The protein profile of the Mariafeld Pinot noir treatments, Riesling treatments and varietal/clonal treatments had similar bands and intensities throughout production. Most juice samples had a very faint/low intensity band around 70 kDa, and more prominent bands between 20-30 kDa. The intensity of the bands decreased over primary fermentation. The origin of the protein bands was not evaluated in this study, however from the literature the identity of the bands can be speculated...
upon. Wine proteins typically range from 10-100 kDa in size (Blasco et al. 2011). TLPs and chitinases are grape pathogenesis-related (PR) proteins and range from 20-30 kDa in size (Meier et al. 2016). Invertase is also another grape-derived protein ranging from 60-65 kDa in size (Blasco et al. 2011). These proteins concentrations decrease during fermentation and in the presence of bentonite (Lira et al. 2014). Mannoproteins have a larger size range from 5-800 kDa and the release of mannoproteins into wine may be a slow process (Pérez-Magarino et al. 2015). The protein bands that formed in the 2.0 g L⁻¹ bentonite Riesling treatments (+/- bentonite in tirage) may be mannoproteins. However, these proteins appeared early on during secondary fermentation and not after the longer lees aging period. Future analysis into protein identification is required to understand the proteins’ origins.

At the beginning of this study, the Bradford assay was used to quantify proteins. Although the Bradford assay provided fast results, pectin interference provided inaccurate results. Furthermore, the BSA standards were lowered to below optimal range from 100 – 1000 µg mL⁻¹ to 10 – 100 µg mL⁻¹ in attempts to capture the protein concentrations found in evaluated samples. The protein concentration was evaluated using an acetone-precipitation method followed by the BCA assay, as well as densitometry from SDS-PAGE gels stained with SYPRO Ruby stain. The BCA assay provided a larger standard quantification range when compared to the Bradford assay. The use of acetone to precipitate the final wine proteins was required to eliminate any interferences from the reducing sugars. When comparing the final protein concentrations with both methods, the concentrations obtained from the BCA assay was consistently lower than values obtained from densitometry. Wine is a complex matrix and evaluating the proteins can be challenging, as there are many interferences in juice and wine and the overall protein concentrations are quite low. In contrast, a study by Vincenzi et al. (2005) discussed the development of a new method to quantify
proteins in wine and compared existing techniques. They concluded that the BCA (Smith) assay provided the most accurate protein results when compared to Lowry and Bradford assay (Vincenzi et al. 2005). They found that wine protein concentrations were significantly higher from acetone-precipitation coupled with BCA (Smith) assay (680.1 ± 111.3 µg mL⁻¹) when compared to densitometry from SDS-PAGE with Coomassie staining (31.8 ± 1.8 µg mL⁻¹). Comparable protein concentrations between the KDS precipitation coupled with BCA assay and densitometry were observed (Vincenzi et al. 2005). The SYPRO Ruby stain is a more sensitive stain compared to the Coomassie stain. Densitometry provides a protein concentration relative to the quantification of the band to which it is being normalized, whereas the BCA and Bradford protein determinations are relative to the BSA standard. For this project, the protein concentrations from densitometry were determined knowing the amount of protein in the 50 kDa band and for the corresponding volume.

The observed trends between samples at different timepoints were similar and the protein profiles also provided a visual understanding of the proteins during sparkling wine production. Methods including FPLC, RP-HPLC and 2D gel electrophoresis can be used to further evaluate proteins in sparkling wine to determine their identification. Each band in an SDS-PAGE gel may be made up of several proteins bands, each with a similar molecular weight. 2D gel electrophoresis would allow us to further separate those proteins.

The excess gushing at disgorging indicated how important the riddling process is to production, as lees that do not settle in the neck and gather on sides of the bottle can cause gushing. This may create nucleation points and increase gushing, resulting in a loss of wine and decrease in foam. Oenological practices such as racking, bentonite addition and yeast selection impact sparkling wine quality (Vanrell et al. 2006; Vincenzi et al. 2010). The yeast performance in secondary
fermentation affects final sparkling wine quality. Yeast viability is heavily dependent on the appropriate tirage buildup as ethanol present in the wine is the main limiting factor affecting fermentation kinetics in bottle (Borrull et al. 2015). The S. bayanus cultures in Chapter 2 did not successfully ferment the base wine into sparkling wine. The initial Saccharomyces cerevisiae EC 1118 Riesling cultures in Chapter 3 were not able to successfully complete Stage 3 of tirage buildup. In both cases, it appears that yeast could not tolerate the ethanol concentration in the base wine which led to increased ethanol stress. Further understanding of the adaptation and proliferation stages of build up is required, as different yeast strains have varying sensitivities to base wine environments.

Along with yeast selection, varietal selection is important to the quality of sparkling wine, as fruit maturity, chemical and protein composition in juice and resulting wine differ amongst varietals. The clones from each varietal evaluated (Chardonnay, Chardonnay musqué, Pinot noir and Riesling) varied in pH, TA and soluble solids. Fruit maturity can impact the harvest date, which can alter the juice composition and quality of sparkling wine (Jones et al. 2014). Soil composition may impact the protein concentrations and resulting foaming properties. All Riesling treatments evaluated in Chapter 4 had the highest protein concentrations at the lees aging midpoint and the protein concentration at this timepoint may influence the foaming properties in the finished sparkling wine.

To grasp a full understanding of the impact of varietal and clonal selection, future protein analysis in the finished wines from both the BCA assay and densitometry are important to investigate. This will provide information on the role different varietals and clones have on protein profiles, concentrations and resulting sparkling wine quality. Further chemical analysis of the finished sparkling wines including pH, TA, ethanol (% v/v), malic acid and residual sugar will provide an
understanding into the chemical compositional changes occurring over the course of sparkling wine production and their effects on sparkling wine quality. The foaming analysis of the finished sparkling wines from the various clonal treatments will aid in determining the role that these treatments have on the foaming properties and overall sparkling wine quality. Along with these bioanalytical techniques, utilizing various sensory science techniques, including difference and discriminative testing, coupled with consumer behaviour analysis of sparkling wines is needed to be able to conclude the best grape varietals, clones and soil compositions suited to quality sparkling wine production in the Niagara Peninsula.
5.1 Literature Cited


Appendix I: Protein Normalization and Quantification from SDS-PAGE gels

Figure 1: Normalization and quantification of proteins using densitometry from SDS-PAGE gels. (A) The total protein signal normalized to the 50kDa band in the reference lane. (B) The total protein signal normalized to the 50kDa band in each lane with increasing volume.
Appendix II: Additional results from Chapter 2

**Figure 1:** Primary fermentation kinetics of soluble solids for control/no treatment and bentonite treated juice inoculated with *S. cerevisiae* EC-1118. Fermentations were performed in four replicates. Soluble solid values represent the average ± standard deviation of the mean from four replicate fermentations.

**Table 1:** Microbial results from yeast colonies present on YPD for all Pinot noir sparkling wine treatments at the lees aging midpoint. The replicates represent the average ± standard deviation of two plates from one bottle. The final colony forming units (CFU mL\(^{-1}\)) represent the average ± standard deviation of all six plates per treatment (three bottles tested in duplicate).

<table>
<thead>
<tr>
<th></th>
<th>CFU mL(^{-1})</th>
<th>Control</th>
<th>Bentonite in <em>tirage</em></th>
<th>Bentonite in juice</th>
<th>Bentonite in juice &amp; <em>tirage</em></th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rep 1</td>
<td>20 ± 14</td>
<td>395 ± 35</td>
<td>0 ± 0</td>
<td>35 ± 7</td>
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<tr>
<td>Rep 2</td>
<td>0 ± 0</td>
<td>50 ± 14</td>
<td>360 ± 85</td>
<td>295 ± 7</td>
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</tr>
<tr>
<td>Rep 3</td>
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<td>0 ± 0</td>
<td>45 ± 49</td>
<td>0 ± 0</td>
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<tr>
<td><strong>Average ± SD</strong></td>
<td>82 ± 127</td>
<td>148 ± 193</td>
<td>135 ± 180</td>
<td>110 ± 144</td>
<td></td>
</tr>
</tbody>
</table>

*S. bayanus* Brock isolate

<table>
<thead>
<tr>
<th></th>
<th>CFU mL(^{-1})</th>
<th>Control</th>
<th>Bentonite in <em>tirage</em></th>
<th>Bentonite in juice</th>
<th>Bentonite in juice &amp; <em>tirage</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rep 1</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td></td>
</tr>
<tr>
<td>Rep 2</td>
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<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td></td>
</tr>
<tr>
<td>Rep 3</td>
<td>5 ± 7</td>
<td>10 ± 0</td>
<td>10 ± 14</td>
<td>0 ± 0</td>
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</tr>
<tr>
<td><strong>Average ± SD</strong></td>
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<td>3 ± 5</td>
<td>3 ± 8</td>
<td>0 ± 0</td>
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</tbody>
</table>
Appendix III: Additional results from Chapter 3

**Figure 1:** Primary fermentation kinetics of soluble solids for control/no treatment, pectinase treatment and bentonite treated juice inoculated with *S. cerevisiae* EC-1118. Fermentations were performed in triplicates. Soluble solid values represent the average ± standard deviation of the mean from four replicate fermentations.

**Table 1:** Microbial results from yeast colonies present on YPD for all Riesling sparkling wine treatments at the lees aging midpoint. The replicates represent the average ± standard deviation of two plates from one bottle. The final colony forming units (CFU mL⁻¹) represent the average ± standard deviation of all six plates per treatment (three bottles tested in duplicate).

<table>
<thead>
<tr>
<th>CFU mL⁻¹</th>
<th>Control BW</th>
<th>Pectinase BW</th>
<th>Bentonite BW</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(-) Bentonite in tirage</td>
<td>(+) Bentonite in tirage</td>
<td>(-) Bentonite in tirage</td>
</tr>
<tr>
<td>Rep 1</td>
<td>120 ± 170</td>
<td>420 ± 14</td>
<td>*TNTC</td>
</tr>
<tr>
<td>Rep 2</td>
<td>35 ± 7</td>
<td>0 ± 0</td>
<td>TN TC</td>
</tr>
<tr>
<td>Rep 3</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>TN TC</td>
</tr>
<tr>
<td>Average ± SD</td>
<td>52 ± 94</td>
<td>140 ± 217</td>
<td>TN TC</td>
</tr>
</tbody>
</table>

*TNTC – Too numerous to count*