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Nutrient Addition to Low pH Base Wines (L. cv. Riesling) during Yeast Acclimatization for Sparkling Wine: Its Influence on Yeast Cell Growth, Sugar Consumption and Nitrogen Usage

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Abstract: In traditional method sparkling wine production, to carry out a successful second alcoholic fermentation, yeast are acclimatized to stressful base wine conditions. Base wines typically have low pH, low nutrient concentrations, high acid concentrations, contain sulfur dioxide (SO_2), and high ethanol concentrations. Supplementing yeast during the acclimatization stages prior to second alcoholic fermentation with different nutrient sources was assessed to determine the impact on yeast growth, sugar consumption and nitrogen usage. Four treatments were tested with *Saccharomyces cerevisiae* strain EC1118: the control (T1) with no additives; addition of diammonium phosphate (DAP) during acclimatization, (T2); Go-Ferm® inclusion during yeast rehydration (GF), (T3); and DAP + GF (T4). Results (n = 4) indicated that supplementing with DAP, GF or DAP + GF increased both the rate of sugar consumption and the concentration of viable cells during the yeast acclimatization phase in comparison to the control. Treatments supplemented with DAP + GF or DAP alone resulted in yeast consuming 228 and 220 mg N/L during the acclimatization phase, respectively. Yeast treated only with GF consumed 94 mg N/L in comparison to the control, which consumed 23 mg N/L. The time required to reach the target specific gravity (1.010) during acclimatization was significantly reduced to 57 h for yeast treated with DAP and GF, 69 h for yeast treated with DAP only and 81 h for yeast rehydrated with GF in comparison to 105 h for the control. Our results suggest that nutrients used during yeast acclimatization could have an important impact on the kinetics of second alcoholic fermentation.

Keywords: nutrients; base wine; second alcoholic fermentation; sparkling wine

1. Introduction

Yeast acclimatization to adapt the yeast to the hostile environment of base wines (i.e., low pH, high acidity and alcohol) for a second alcoholic fermentation in sparkling wine production (traditional method) involves three stages: rehydration (stage 1), adaptation (stage 2) and proliferation (stage 3) [1,2]. At the end of proliferation, the acclimated culture is better conditioned to survive the stressful conditions of the second alcoholic fermentation. Yeast performance during second alcoholic fermentation can potentially be further optimized by the addition of micronutrients during yeast rehydration [3] and/or a source of yeast assimilable nitrogen (YAN) during adaptation and/or proliferation [4]. Yeast can be adapted to the conditions of the second alcoholic fermentation using an acclimatization process, often

referred to as *tirage*. *Liqueur de Tirage* is achieved by using active dry yeast (ADY), base wine, water and wine-based solution with 500 g/L of sugar to adapt the yeast to an environment with a low pH, a high ethanol concentration, sulfur dioxide (SO_2), carbon dioxide (CO_2) and pressure [2,5]. *Saccharomyces cerevisiae* is a strong fermenter, but some yeast strains can still struggle to grow in a medium with low nutrient availability, low pH, high ethanol concentration and high pressure [5].

The traditional method (as with the charmat and transfer methods) produces wine that is carbonated by a second alcoholic fermentation, initiated by increasing the base wine sugar concentration to approximately 24 g/L (depending on wine style), then bottling and inoculating it with the acclimatized yeast, also known as the *liqueur de tirage* (LT) [6]. This solution contains 60 to 80 million yeast cells per mL of wine as well as sugar, base wine, and usually a riddling agent to help with yeast flocculation/settling [2,7]. Typical base wine conditions can include free SO_2 concentrations of up to 20–30 mg/L, total SO_2 concentrations of up to 60 mg/L, ethanol concentrations reaching as high as 11.5% (*v*/*v*), and low pH conditions ranging from 2.8 to 3.3 [6]. The yeasts are acclimatized by the rehydration and continuous feeding of active dry yeast (ADY) to achieve a sustained growth phase [8]. This continuous growth phase is accomplished in three stages known as rehydration (stage 1), adaptation (stage 2), and proliferation (stage 3) [1,2].

The majority of yeast assimilable nitrogen (YAN mg N/L) of grape will typically be assimilated during the primary fermentation depending on the temperature of primary fermentation, yeast strain used and the sugar level. Normally, ammonia is fully assimilated, and amino acids will make up the remaining YAN in the base wine when the grape has an optimal YAN concentration [9]. The source and timing of YAN addition during the production of sparkling wine impacts the fermentation kinetics and completion of the second alcoholic fermentation [4,10]. Augmenting the yeast with YAN can provide yeast with sufficient nitrogen to complete a second alcoholic fermentation even in base wines deprived of nitrogen [11]. The source of nitrogen used to supplement the yeast impacts yeast growth during yeast acclimatization. Inorganic nitrogen (i.e., diammonium phosphate-based products (DAP)) has been reported to favor fast growth during yeast acclimatization but produced a wine with a lower final pressure [4]. However, cultures treated with organic nitrogen were found to have a relatively slower growth rate but produced a wine with a higher final pressure [11].

Nutrient additions during the yeast acclimatization stages have been shown to provide sufficient nutrients for the completion of the second alcoholic fermentation [10]. However, the increase in fermentation kinetics may be counteracted if the yeast acclimatization process is supplemented with organic, or inorganic nitrogen, or if the base wine has a YAN concentration exceeding 30 mg N/L [4,10]. *S. cerevisiae* is reported to require between 20 and 30 mg N/L of YAN in the base wine in order to complete the second alcoholic fermentation in bottle [4,12]. The nitrogen requirement for the second alcoholic fermentation is much lower than the primary fermentation. This is due to less sugar being consumed and reduced cell growth as the yeast will normally only replicate two to three times during the second alcoholic fermentation, which has been successful with a YAN concentration of 20 mg N/L [12,13]. Several authors suggest that supplementing yeast during the acclimatization stages with nitrogen is more beneficial than supplementing the base wine to produce a fermentative advantage during the second alcoholic fermentation since ammonia or amino acids are more readily assimilated by yeast during the acclimatization process [14,15]. The quantity and timing of diammonium phosphate (DAP) addition is important, as revealed by Laurent and Valade [1]. These authors showed that supplementing during the adaptation and proliferation stages is more beneficial for yeast growth during *tirage* than a single addition during the adaptation stage. However, to the best of our knowledge, no research has studied the impact of a dual addition of inorganic nitrogen (DAP) during acclimatization in conjunction with a yeast micronutrient addition during yeast rehydration that provides organic nitrogen as well.

Yeast survival factors are membrane-associated fatty acids and sterols that help improve the ethanol tolerance of yeast. These survival factors are necessary in order to ensure a complete fermentation [3]. Oxygen is essential for the yeast to synthesize survival factors. However, survival factors can also be provided by the addition of yeast hulls to the medium [16,17].

Oxygenating the medium during yeast rehydration for the primary fermentation or during yeast acclimatization enables yeast to synthesize their own survival factors. However, in the presence of excess oxygen, reactive oxygen species (ROS) can form and have a detrimental impact on the yeast's survival [5]. ROS can cause function-altering damage to DNA, proteins, and lipids [18]. Oxidative damage to lipids can increase membrane fluidity by shortening the fatty acyl chains, while proteins damaged by ROS can become inactivated [18]. This makes it important not to excessively oxygenate yeast during acclimatization when preparing the medium for a second alcoholic fermentation.

Second alcoholic fermentation of sparkling wine is conducted in a reductive environment with little oxygen available. Therefore, in order for the fermentation to be successful, the yeast must have adequate survival factors prior to inoculation for the second alcoholic fermentation. Yeast acclimatization conducted with a 20% headspace or daily oxygenation of 50 mg/L has been shown to provide sufficient oxygenation to complete a second alcoholic fermentation [2,5]. Alternatively, yeast hulls can be incorporated during acclimatization to provide these survival factors, decreasing the need for oxygenation [3,19]. The addition of yeast hulls may increase the yeast biomass present in the bottle at the end of the second alcoholic fermentation, which in turn, may lead to riddling and/or disgorging issues due to a higher level of sediment in the bottle neck.

Autolyzed yeasts are commonly used as a source of micronutrients providing different nutrients to the fermentation based on its level of processing. Autolyzed yeasts can be processed into inactive yeasts, yeast autolysates, yeast extracts and yeast hulls [3]. Yeast hulls provide the previously mentioned survival factors, while yeast extract supplements provide the yeast with important micronutrients such as magnesium, manganese, zinc, copper, iron, potassium, and calcium, as well as vitamins such as thiamine, riboflavin, niacin, pyridoxine, biotin, pantothenic acid and inositol [3,20]. Some of these micronutrients such as; zinc [21], potassium (K) [22], biotin and thiamine [23], have a direct impact on the performance of yeast under fermentative conditions. Thiamine, and to a lesser degree biotin, have been shown to increase the viability of yeast by up to 30% [23]. Zinc is an important cofactor for several proteins that increase ethanol tolerance [21], while K helps the cell to regulate its internal pH. The addition of degraded yeast autolysates to the acclimatization has also been shown to beneficially affect sparkling wine by decreasing the period of time on lees required to obtain optimal aroma and bubble quality [7]. Other factors that have been assessed for their ability to influence the second alcoholic fermentation are the yeast strain used [24] in addition to supplementing either the yeast during acclimatization, or the base wine with nitrogen. Varying the level of oxygen and sugar available to the yeast during acclimatization has also been found to influence second alcoholic fermentation [1,5,11].

The aim of this study was to compare the impact of different sources of YAN and micronutrients on the kinetics of yeast acclimatization prior to inoculation for second alcoholic fermentation. Riesling base wine was used for this study, as it has become commonly used in Ontario, Canada for the production of Traditional Method and Charmat/Tank Method sparkling wines. Nitrogen supplementation with DAP, yeast rehydration with the micronutrient Go-Ferm®, or a combination of the two were trialed during the yeast acclimatization procedure to assess the impact on yeast growth, sugar consumption and nitrogen usage during acclimatization.

2. Materials and Methods

2.1. Yeast Strain

S. cerevisiae strain EC1118 was provided by Lallemand Inc. (Montreal, QC, Canada). The chemical composition of the Riesling base wine is detailed in Table 1.

Table 1. Chemical composition of the Riesling base wine.

Titratable Acidity (g/L Tartaric)		7.3
pH		2.85
YAN *	Ammonia (mg N/L)	<7
	Alpha-amino acids (mg N/L)	26.8
Sugar	Sucrose (g/L)	<0.09
	Fructose (g/L)	0.25
	Glucose (g/L)	<0.09
Ethanol (% (*v*/*v*))		10.6
SO_2	Free (ppm)	8.4
	Total (ppm)	56.5

* YAN = yeast assimilable nitrogen.

2.2. Chemical Analysis

Assay kits from Megazyme (Bray, Ireland) were used to measure the concentration of sucrose, fructose and glucose (K-SUFRG), ammonia (K-AMIAR) and alpha amino acids (K-PANOPA). Procedures for enzyme kits were conducted following manufacturer's instructions, with volumes for all reagents divided by two. Ethanol concentration was analysed using gas chromatography with flame ionization detection (GC-FID) following the method of Nurgel et al. [25]. Free and total SO_2 concentrations were measured using the aspiration method [26]. pH was measured using a pH meter that had been calibrated to a pH of 4, 7 and 10. Titratable acidity (TA g/L) was measured by titrating the wine with 0.1 N NaOH to a pH endpoint of 8.2.

2.3. Yeast Acclimatization/Tirage

Yeast acclimatization was conducted according to the protocol used by the Cool Climate Oenology and Viticulture Institute (CCOVI), Brock University, Ontario, Canada. This protocol is detailed in Figure 1, involving three stages: rehydration (stage 1), adaptation (stage 2) and proliferation (stage 3).

Yeast was rehydrated during rehydration (stage 1) for 30 min, with stirring at 15 min and 30 min. The adaptation stage was carried out in 18 h to reach a target specific gravity of 1.030. The proliferation was performed for 87 h to reach a target specific gravity of 1.010. Details of the volumes used at each stage are listed in Table 2. The liqueur solution contains 500 g/L sugar.

Table 2. Quantity of nutrients added during the three stages of yeast acclimatization.

Stage	Supplement	Control (T1)	DAP * (T2)	Go-Ferm® (T3)	DAP + Go- Ferm® (T4)
Rehydration (60 mL volume)	Go-Ferm® (g)	0	0	7.5	7.5
Adaptation (180 mL volume)	DAP (mg)	0	360	0	360
Proliferation (1800 mL volume)	DAP (mg)	0	600	0	600

* DAP = diammonium phosphate.

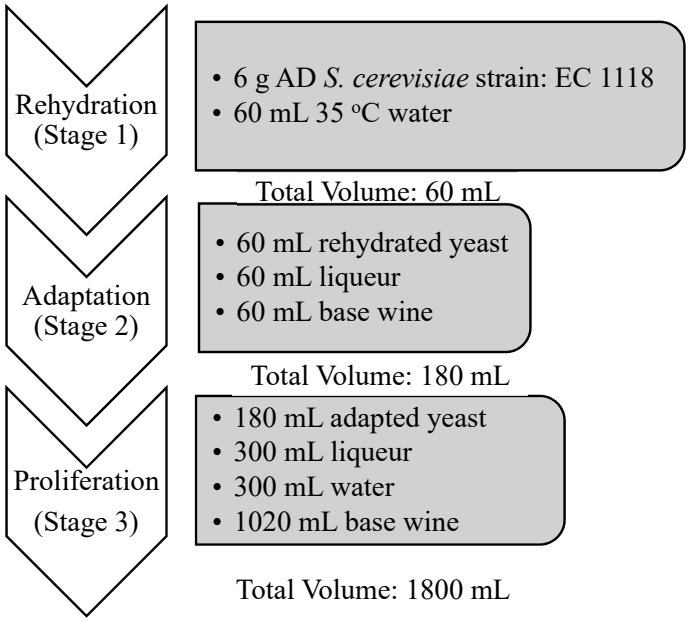

Figure 1. The yeast acclimatization protocol.

The four treatments with four replicates were the control with no additions during the acclimatization (T1); DAP addition during stage 2 (adaptation) and stage 3 (proliferation) (DAP), (T2); Go-Ferm® used for yeast rehydration (GF), (T3); and a combination of DAP addition + GF during rehydration (T4). The rate of GF used during rehydration (stage 1) was in accordance with the manufacturer's recommended rate of 1.25 times the mass of yeast rehydrated. Additions of DAP during adaptation (2 g/L, stage 2) and proliferation (333 mg/L, stage 3) were at the rate defined by Valade and Laurent [2].

The treatments were monitored every eight hours during adaptation, then every twelve hours during proliferation. Sugar consumption was measured by specific gravity hydrometry, temperature was measured using a thermometer, while viable cell concentration and total cell concentrations were differentiated using methylene blue and counted using a haemocytometer as previously reported [27]. The temperature during the adaptation and proliferation stages ranged between 23 and 26 °C. The concentrations of primary amino acids and ammonia were determined using ammonia (K-AMIAR) and alpha amino acids (K-PANOPA) kits from Megazyme (Bray, Ireland) on samples collected at the start and end of the adaptation stage and proliferation stage.

2.4. Statistical Analysis

Analysis of variance (ANOVA) with mean separation by Fisher's Protected Least Significant Difference (LSD) test ($p < 0.05$) was carried out using XLSTAT statistical software package released by Addinsoft (Version 7.1; New York, NY, USA).

3. Results and Discussion

3.1. Nutrient Addition Impact on Cell Growth and Viability During Yeast Acclimatization

There were no significant differences between treatments for viable cell concentration during the adaptation stage 2 of cell acclimatization (Figure 2a). During the proliferation stage 3, DAP (T2) and GF (T3) treatments reached viable cell concentrations at 69, 71, 93 and 105 h that were significantly higher than the control ($p < 0.05$) while the combined DAP + GF (T4) treatment reached viable cell concentrations significantly greater than all treatments for these time points ($p < 0.05$) (Figure 2b). Supplementing yeast with DAP (T2) stimulated cell growth, providing them with a source of easily usable ammonia nitrogen [28,29]. The DAP supplemented yeast required 69 h to

reach a viable cell concentration that was significantly higher than the control ($p < 0.05$) (Figure 2b). The extended period of time required for the DAP supplement to impact the viable cell concentration later in stage 3 may be due to the yeast acclimatization procedure beginning with a high viable cell concentration [1]. It has previously been shown that there is a maximum viable cell concentration attainable in a *tirage* (acclimatization) medium. A *tirage* inoculated with 12 g/L ADY typically leads to marginal growth during adaptation in stage 2 and one doubling during the proliferation stage 3 [1]. For this experiment, the quantity of yeast rehydrated and added to stage 2 was equal to a concentration of 33 g/L (Figure 1) whereas the yeast at the start of stage 3 would be further diluted 10-fold. Since the quantity of yeast rehydrated for the acclimatization equaled the maximum cell concentration that the medium could sustain, the culture may have been prevented from significantly increasing its viable cell concentration during stage 2. GF has previously been shown to increase the concentration of viable cells under stressful conditions [3,30]. This observation was supported by our experiments as rehydrating the yeast in GF (T3) led to a higher viable cell concentration at the end of stage 3 in comparison to the control ($p < 0.05$) (Figure 2b). GF can provide essential micronutrients including zinc, potassium, biotin and thiamine, which have all shown to increase the tolerance of yeast to base wine conditions [3,20]. The viable cell concentration for the acclimatization supplemented with DAP and GF (T4) was significantly higher than all other treatments for most of the proliferation stage (stage 3) ($p < 0.05$) (Figure 2b). The decrease in the viable cell concentration at the end of the acclimatization steps may be due to the yeast exhausting its supply of fermentable sugars (Figure 3b). Cell viability was higher for the DAP + GF treatment than the control at 105 h but not significantly different from the GF or DAP treatments ($p < 0.05$).

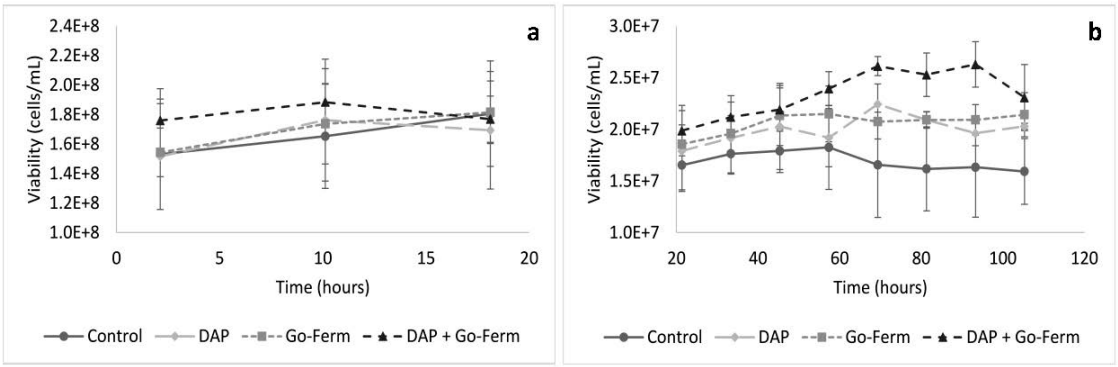

Figure 2. Cell Viability during yeast acclimatization. Mean concentration of viable cells during the adaptation stage 2 (**a**) and proliferation stage 3 (**b**) were measured and recorded as units of 10^8 cells/mL (a) or 10^7 cells/mL (b) Error bars represent the standard deviation of $n = 4$ replicates.

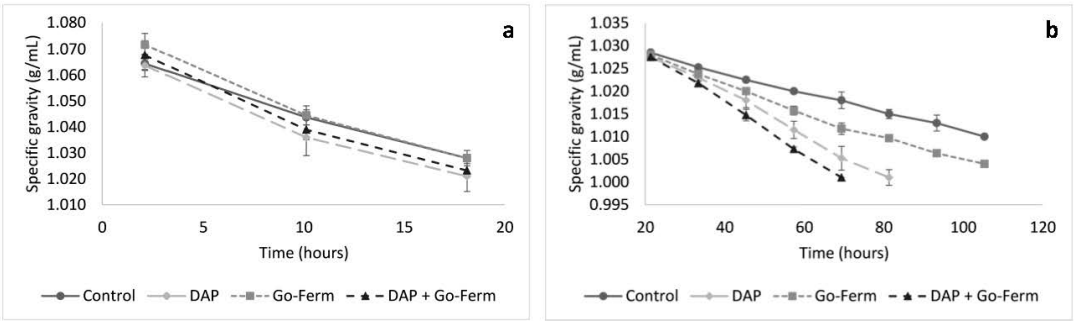

Figure 3. Sugar consumption during yeast acclimatization. Mean sugar consumption throughout yeast acclimatization represented as a decrease in specific gravity during the adaptation stage 2 (**a**) and proliferation stage 3 (**b**). Error bars represent the standard deviation of $n = 4$ replicates.

3.2. Nutrient Addition Impact on Sugar Consumption During Yeast Acclimatization

All nutrient supplements had a similar but faster rate of sugar consumption during the adaptation stage 2 compared to the control (Figure 3a, Table 3). However, the rate of sugar consumption varied significantly ($p < 0.05$) during the proliferation stage 3 depending on the treatment (Figure 3b, Table 3). The DAP + GF (T4) treatment stimulated sugar consumption the greatest, allowing cells to reach the target specific gravity below 1.010 in only 57 h, signaling the end of stage 3 (Figure 3b). DAP alone (T2) showed the next fastest sugar consumption rate reaching the specific gravity target in 69 h, followed by GF (T3) in 81 h with the control requiring 105 h to reach this target, with a sugar consumption rate half that found for the DAP + GF (T4) treatment (Figure 3b, Table 3). The rate of sugar consumption was slower for the acclimatization supplemented only with GF than the one supplemented only with DAP, likely due to differences in nitrogen composition and quantity. Contrary to what was observed for cell growth, DAP had an immediate impact on the rate of sugar consumption, indicating that at the cell concentrations observed during yeast acclimatization, ammonia impacts the rate of sugar consumption more than stimulates cell growth (Figure 2 versus Figure 3).

Table 3. Rate of sugar consumption during yeast acclimatization calculated from the difference in time and specific gravity at the start and end of adaptation stage 2 and proliferation stage 3, respectively. Treatments were replicated ($n = 4$) and are represented as the mean ± standard deviation. Lower case letters indicate statistical differences in sugar consumption rates between treatments determined by ANOVA with mean separation by Fisher's Protected (LSD0.05).

	Rate of Sugar Consumption (mg/mL/hour)	
	Adaptation Stage 2	**Proliferation Stage 3**
Control	2.3 ± 0.2 ^{b}	0.23 ± 0.01 ^{d}
DAP	2.7 ± 0.1 ^{a}	0.45 ± 0.01 ^{b}
Go-Ferm®	2.7 ± 0.1 ^{a}	0.31 ± 0.01 ^{c}
DAP + Go-Ferm®	2.8 ± 0.3 ^{a}	0.55 ± 0.01 ^{a}

3.3. Nutrient Addition Impact on Nitrogen Consumption During Yeast Acclimatization

The quantity of nitrogen assimilated by the yeast during acclimatization was directly proportional to the type of nutrients the yeast were supplemented with (Figure 4, Table 4). The DAP and GF both provided the media with nitrogen whereby DAP increased the ammonia nitrogen, and GF increased the amino acid nitrogen (Figure 4). Yeasts in the DAP and the DAP + GF treatments assimilated the most nitrogen at 220 and 228 mg N/L, respectfully, followed by the yeast rehydrated with GF assimilating 94 mg N/L, leaving the yeast without any supplementation (T1) consuming only 23 mg N/L (Table 4). The only YAN available for the yeast in the control treatment (T1) was from the amino acids present in the base wine (Table 1) and the *liqueur de tirage* and the amino acids released from rehydrated yeast cells. During rehydration (stage 1), amino acids likely leached out of the yeast [31] increasing the amino acid concentration of the media used for adaptation. This could explain the initial spike in amino acids observed in Figure 4 for the control (T1) and DAP (T2) treatments at the start of adaptation (stage 2) in comparison to what was available in the base wine. The control treatment (T1) subsequently assimilated the amino acids while the yeast cells supplemented with DAP (T2) prioritized the assimilation of ammonia and continued to leach out amino acids during the adaptation stage (stage 2) (Figure 4). Rehydrating yeast in GF (T3) provided them with micronutrients as well as YAN in the form of amino acids (Figure 4). Yeast supplemented with GF (T3) during rehydration assimilated 80 ± 10 mg N/L during stage 2 out of the 240 ± 10 mg N/L amino acids available at the start of the adaptation stage 2 and then subsequently assimilated another 14 mg N/L during stage 3. Supplementing the yeast with DAP (T4) repressed the yeast's ability to assimilate amino acids (Figure 4), illustrating that ammonia is the preferred form of nitrogen for *S. cerevisiae* [23]. Yeast supplemented with both DAP and GF (T4) during the acclimatization stages did not assimilate a

significant quantity of amino acids. Similarly, rehydrating the yeast in GF did not impact their ability to assimilate ammonia, since yeast supplemented with DAP (T2) and yeast supplemented with both DAP and GF (T4) assimilated similar amounts of nitrogen at 220 and 228 mg N/L, respectively, during acclimatization (Figure 4, Table 4).

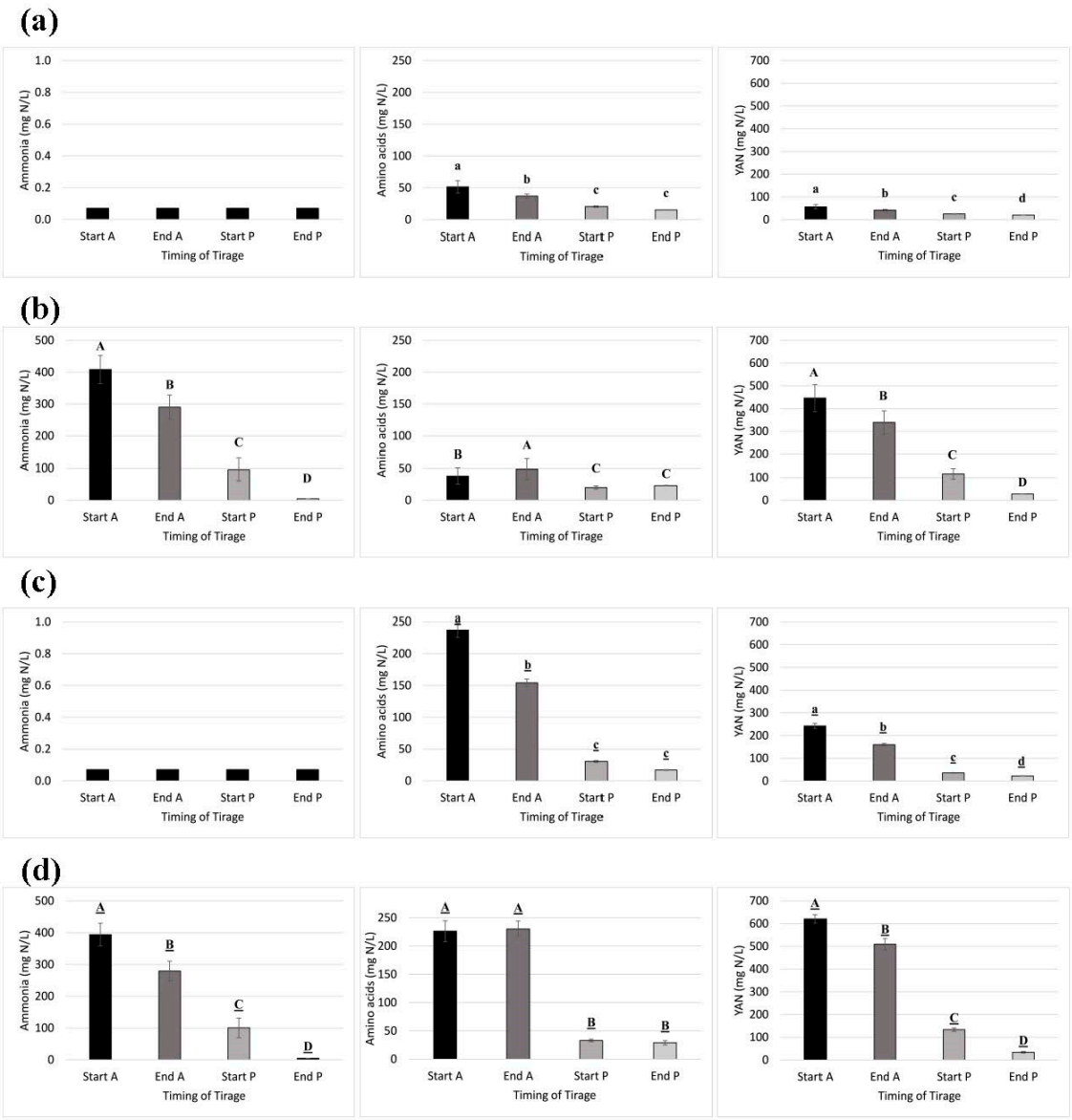

Figure 4. Nitrogen concentration during the stages of yeast acclimatization in the form of ammonia nitrogen (**column 1**), amino acid nitrogen (**column 2**) and Total YAN (**column 3**), all reported as mg N/L. Nitrogen from ammonia and amino acids were determined at the start (Start A) and end (End A) of adaptation stage 2 and the start (Start P) and end (End P) of proliferation stage 3 for all four treatments. Total YAN is the addition of ammonia and amino acid nitrogen. Treatments were: T1 control, panel (**a**); T2 DAP addition, panel (**b**); T3 Go-Ferm® addition, panel (**c**); and T4 DAP + Go-Ferm® addition, panel (**d**). Due to low ammonia levels in T1 and T3, reported at the limit of detection of the kit (0.071 mg N/L), the Y-axis scale for ammonia was resized to range from 0 to 1 mg N/L for panel (**a**) and (**c**). All other scales were kept constant between treatments for comparison purposes. Different letters within a treatment for a given nitrogen source refers to significant differences ($p < 0.05$) in nitrogen concentration between time points as determined by ANOVA with mean separation by Fisher's Protected (LSD0.05).

Table 4. Quantity of total nitrogen assimilated during adaptation, proliferation and the entire yeast acclimatization process. Treatments were replicated (n = 4) and are represented as the mean ± standard deviation. Lower case letters indicate statistical differences in nitrogen consumption between treatments determined by ANOVA with mean separation by Fisher's Protected (LSD0.05).

Treatment	Nitrogen Assimilated (mg N/L)		
	Adaptation	Proliferation	Total
Control	18 ± 4 ^{c}	5 ± 1 ^{b}	23 ± 3 ^{c}
DAP	120 ± 30 ^{a}	100 ± 20 ^{a}	220 ± 30 ^{a}
Go-Ferm®	80 ± 10 ^{b}	14 ± 1 ^{b}	94 ± 9 ^{b}
DAP + Go-Ferm®	110 ± 20 ^{a}	108 ± 5 ^{a}	228 ± 20 ^{a}

3.4. Overall Impact of Yeast Acclimatization with or without Nutrients Prior to Second Alcoholic Fermentation

The yeast selected for this experiment (*S. cerevisiae* strain EC1118) successfully completed the acclimatization process without any further additions, reaching target specific gravities during the adaptation stage (1.030 g/mL) and the proliferation stage (1.010 g/mL) indicating that the acclimatization protocol worked. The ability of EC1118 to complete the acclimatization process without any supplementation indicated that the 26.8 mg N/L present in the base wine was sufficient in this media for this yeast strain, supporting other reports that 20 mg N/L in base wine provides sufficient YAN [12]. Yeast strain has been shown to greatly affect the fermentation kinetics of the second alcoholic fermentation [4]. Using a base wine that does not meet the minimum 20 mg N/L would better illustrate the impact that supplementing YAN during acclimatization has on yeast performance [4,12]. Other parameters that would be important to assess are the impact that base wine pH, varied concentrations of SO_2 and alcohol levels have on the benefits of supplementing the yeast during acclimatization. Additionally, the yeast sediment size from supplementation treatments, upon the second alcoholic fermentation completion, requires measuring to assess any potential issues that the winemaker could encounter from large yeast deposits that could occur during the riddling and disgorging stages of bottle-fermented sparkling wine.

Base wine that has 20–30 mg N/L does not require supplementing with micronutrients and/or inorganic nitrogen during yeast acclimation even with a low pH of 2.85 as in this study. DAP had a larger impact on the rate of sugar consumption during proliferation. Meanwhile, DAP + GF had a synergistic effect on yeast growth during proliferation, further increasing the concentration of viable cells and the rate of sugar consumption when the acclimatization was supplemented with both. While supplementing with DAP (T2) has more of an impact on the rate of sugar consumption, supplementing with GF (T3) has more of an impact on the viable cell concentration.

The impact of these acclimatization procedures on the success of the second alcoholic fermentation, bubble stability and final sensory profile of sparkling wine should be investigated. This will determine whether a larger yeast biomass that consumes sugar faster during acclimatization is beneficial, detrimental or has no impact for sparkling wine production or if the larger yeast biomass has an impact on the sensory profile of the wine during aging on yeast lees and post-disgorging. It is possible that improving the fermentation kinetics during acclimatization does not guarantee improved fermentation kinetics during the second alcoholic fermentation. Marti-Raga [4] reported that slower growth rates during acclimatization produced a sparkling wine with a higher final pressure.

Further research regarding the impact of supplementing yeast during acclimatization with YAN and micronutrients on the second alcoholic fermentation is required. Determining the impact of different variables on yeast performance during the second alcoholic fermentation, i.e., yeast strain, base wine composition and the rate and type of YAN supplemented during acclimatization are currently under investigation.

4. Conclusions

Our study was aimed at quantifying the impact that supplementing yeast with DAP and/or Go-Ferm® had on yeast performance during acclimatization of yeast cultures to prepare them for second alcoholic fermentation. Supplementing yeast with vitamins and minerals (Go-Ferm®) improved yeast viability during yeast acclimatization. Supplementing the acclimatization culture with DAP increased the rate of sugar consumption and stimulated yeast growth ($p < 0.05$). Supplementing with both DAP and Go-Ferm® had a synergistic impact on the rate of sugar consumption and yeast growth, reducing the time to build up the culture for inoculation 2-fold from 105 h to only 57 h ($p < 0.05$). The impact of these acclimatization procedures on the second alcoholic fermentation and downstream sparkling wine processes are required to determine whether higher yeast concentrations and a faster acclimatization are beneficial for sparkling wine production, and resultant sparkling wine flavour.

Author Contributions: B.K. and D.L.I. conceived and designed the experiments. J.P. performed the fermentations and did all laboratory and statistical analysis. B.K., J.P. and D.L.I. contributed to writing of the manuscript. B.K. and D.L.I. reviewed, edited and formatted the manuscript. All authors have read and agreed to the published version of the manuscript.

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References

1. Laurent, M.; Valade, M. La Préparation du Levain a Partir de Levures Seches Actives. *Le Vign Champen Mars* **2007**, 74–95.
2. Valade, M.; Laurent, M. La Réussite de la Prise de Mousse 2. *Le Vign Champen Mai* **2015**, 54–71.
3. Pozo-Bayón, A.; Andújar-Ortiz, I.; Moreno-Arribas, V. Scientific evidences beyond the application of inactive dry yeast preparations in winemaking. *Food Res. Int.* **2009**, *42*, 754–761. [CrossRef]
4. Marti-Raga, M.; Marullo, P.; Beltran, G.; Mas, A. Nitrogen modulation of yeast fitness and viability during sparkling wine production. *Food Microbiol.* **2016**, *54*, 106–114. [CrossRef]
5. Borrull, A.; López-Martínez, G.; Miró-Abella, E.; Salvadó, Z.; Poblet, M.; Cordero-Otero, R.; Rozès, N. New insights into the physiological state of *Saccharomyces cerevisiae* during ethanol acclimation for producing sparkling wines. *Food Microbiol.* **2016**, *54*, 20–29. [CrossRef]
6. Kemp, B.; Alexandre, H.; Robillard, B.; Marchal, R. Review: Effect of Production Phase on Bottle-Fermented Sparkling Wine Quality. *J. Agric. Food Chem.* **2015**, *63*, 19–38. [CrossRef]
7. Carrascosa, A.V.; Martinez-Rodriguez, A.; Cebollero, E.; González, R. *Saccharomyces* Yeasts II: Secondary Fermentation. In *Molecular Wine Microbiology*; Elsevier: Burlington, MA, USA, 2011; pp. 33–49.
8. Esteve-Zarzoso, B.; Martinez, M.; Rubires, X.; Yuste-Rojas, M.; Torres, M. Applied Wine Microbiology. In *Molecular Wine Microbiology*; Elsevier: Burlington, MA, USA, 2011; pp. 341–355.
9. Margalit, Y. Must and Wine Composition: Nitrogen. In *Concepts in Wine Chemistry*, 3rd ed.; The Wine Appreciation Guild: San Francisco, CA, USA, 2012.
10. Marti-Raga, M.; Sancho, M.; Marullo, P.; Guillamon, J.M.; Mas, A.; Beltran, G. Factors Affecting the Second Fermentation Development: The Role of Nitrogen on Sparkling Wine Production. In *Cava Challenges:.OENOVITI INTERNATIONAL network*, Proceedings of the 33rd International CAVA Congress, Sant Sadurni d'Anoia, Spain, 20 May 2015; Available online: https://en.calameo.com/books/003503752313ba0bf59de (accessed on 14 February 2020).
11. Marti-Raga, M.; Sancho, M.; Guillamon, J.M.; Mas, A.; Beltran, G. The effect of nitrogen addition on the fermentative performance during sparkling wine production. *Food Res. Int.* **2015**, *67*, 126–135. [CrossRef]
12. Valade, M.; Laurent, M.; Moncomble, D. La Réussite de la Prise de Mousse 1. *Le Vign Champen Avril* **2015**, 48–63.

13. Martinez-Moreno, R.; Morales, P.; Gonzalez, R.; Mas, A.; Beltran, G. Biomass production and alcoholic fermentation performance of *Saccharomyces cerevisiae* as a function of nitrogen source. *FEMS Yeast Res.* **2012**, *12*, 477–485. [CrossRef]
14. Leao, C.; van Uden, N. Effects of ethanol and other alkanols on the ammonium transport system of *Saccharomyces cerevisiae*. *Biotechnol. Bioeng. XXV* **1983**, 2085–2089. [CrossRef]
15. Leao, C.; van Uden, N. Effect of ethanol and other alkanols on the general amino acid permease of *Saccharomyces cerevisiae*. *Biotechnol. Bioeng. XXVI* **1984**, 403–405. [CrossRef] [PubMed]
16. Fugelsang, K.C.; Edwards, C.G. *Wine Microbiology Practical Applications and Procedures*; Springer: New York, NY, USA, 2010.
17. Borrull, A.; Poblet, M.; Rozes, N. New insights into the capacity of commercial wine yeasts to grow on sparkling wine media. Factor screening for improving wine yeast selection. *Food Microbiol.* **2015**, *48*, 41–48. [CrossRef] [PubMed]
18. Moradas-Ferreira, P.; Costa, V.; Piper, P.; Mager, W. The molecular defences against reactive oxygen species in yeast. *Mol. Microbiol.* **1996**, *19*, 651–658. [CrossRef] [PubMed]
19. Munoz, E.; Ingledew, W.M. Effect of yeast hulls on stuck and sluggish wine fermentations: Importance of the lipid component. *Appl. Environ. Microbiol.* **1989**, *55*, 1560–1564. [CrossRef]
20. Julien-Ortiz, A.; Dulan, L. Integrare la composizione dei mosti. *Vignevini Riv. Ital. di Vitic e di Enol.* **2002**, *29*, 65–67.
21. Zhao, X.Q.; Bai, F.W. Zinc and yeast stress tolerance: Micronutrient plays a big role. *J. Biotechnol.* **2012**, *158*, 176–183. [CrossRef]
22. Kudo, M.; Vagnoli, P.; Bisson, L.F. Imbalance of pH and potassium concentration as a cause of stuck fermentations. *Am. J. Enol. Vitic.* **1998**, *49*, 295–301.
23. Ribéreau-Gayon, P.; Dubourdieu, D.; Donèche, B.; Lonvaud, A. *Handbook of Enology: Volume 1, The Microbiology of Wine and Vinifications*, 2nd ed.; John Wiley & Sons, Ltd.: Chichester, UK, 2006.
24. Benucci, I.; Liburdi, K.; Cerreti, M.; Esti, M. Characterization of active dry wine yeast during starter culture (pied de cuve) preparation for sparkling wine production. *J. Food Sci.* **2016**, *81*, M2015–M2020. [CrossRef]
25. Nurgel, C.; Pickering, G.; Inglis, D. Sensory and chemical characteristics of Canadian Icewines. *J. Sci. Food Agric.* **2004**, *84*, 1675–1684. [CrossRef]
26. Rankine, B.C.; Pocock, K.J. Alkalimetric determination of sulfur dioxide in wine. *Aust. Wine Brew Spirit Rev.* **1970**, *88*, 40–44.
27. Muysson, J.; Miller, L.; Allie, R.; Inglis, D.L. The Use of CRISPR-Cas9 Genome Editing to Determine the Importance of Glycerol Uptake in Wine Yeast during Icewine Fermentation. *Fermentation* **2019**, *5*, 93. [CrossRef]
28. Bisson, L.F. Stuck and Sluggish Fermentation. *Am. J. Enol. Vitic.* **1999**, *50*, 107–119.
29. Bell, S.J.; Henschke, P.A. Implications of nitrogen nutrition for grapes, fermentation and wine. *Aust. J. Grape Wine Res.* **2005**, *11*, 242–295. [CrossRef]
30. Kontkanen, D.; Inglis, D.L.; Pickering, G.J.; Reynolds, A. Effect of Yeast inoculation rate, acclimatization, and nutrient addition on icewine fermentation. *Am. J. Enol. Vitic.* **2004**, *55*, 363–370.
31. Ponte, J.G.; Glass, R.L.; Geddes, W.F. Studies on the behavior of active dry yeast in breadmaking. *Cereal Chem.* **1960**, *37*, 263–279.