Influence of Must Supplementation on Growth of *Pediococcus* spp. after Alcoholic Fermentation

M.E. Wade[1], J.P. Osborne[2], C.G. Edwards[*,1]

(1) School of Food Science, Washington State University, Pullman, WA, USA 99164-6376
(2) Department of Food Science and Technology, Oregon State University, Corvallis, OR, USA 97331

Submitted for publication: September 2018
Accepted for publication: November 2018

Key words: *Pediococcus*, nutrients, yeast assimilable nitrogen, wine spoilage

One factor potentially affecting growth of wine spoilage microbes (e.g., *Pediococcus* spp.) is the presence of nutrients not consumed during alcoholic fermentation by *Saccharomyces cerevisiae*. To assess the impact of must nutrient supplementation on *Pediococcus* spp., synthetic grape musts containing low (55.2 mg N/L), medium (250 mg N/L), or high (530 mg N/L) concentrations of yeast assimilable nitrogen (YAN) were fermented by *S. cerevisiae*. Upon cessation of fermentative activity *P. damnosus* OW-2, *P. inopinatus* OW-8, *P. parvulus* WS-7C, WS-29A, OW-1, or *P. pentosaceus* ATCC 33316 were inoculated at 10^4 to 10^5 cfu/mL. With the exceptions of OW-1 and OW-2, none of the other species or strains grew in the synthetic wines unless yeast extract or peptone was added, suggesting the absence of an essential nutrient. Experiments were replicated using Cabernet Sauvignon musts containing low (66.9 mg N/L), medium (219 mg N/L), and high (438 mg N/L) YAN. In general, wines containing the greatest residual amino acid concentrations (high YAN) supported better growth of the aforementioned *Pediococcus* spp. However, low YAN wines containing negligible residual nitrogen achieved similar populations after a short period of initial inhibition, suggesting that 'excessive' nitrogen supplementation to musts does not have a large impact on growth of pediococci post alcoholic fermentation.

INTRODUCTION

Generally considered a spoilage microorganism in winemaking (Lonvaud-Funel, 1999), *Pediococcus* spp. have been isolated throughout vinification, especially during wine aging (Wade *et al.*, 2018). More recently, it has been argued that the frequencies of infections may be increasing (Wade *et al.*, 2018), potentially due to changes to viticulture conditions which impact must composition. As an example, the higher pH of grape musts commonly associated with current viticulture practices (Mira de Orduña, 2010) favors growth of *Pediococcus* spp. as these bacteria dominate microflora in wines above pH 3.5 (Wibowo *et al.*, 1985; Davis *et al.*, 1986). Furthermore, at pH >3.5, the antimicrobial form of SO_2 (*i.e.*, molecular SO_2 or $SO_2 \bullet H_2O$) is in less abundance, thereby increasing the risk of spoilage (Fugelsang & Edwards, 2007).

Besides higher pH, modern grape musts also tend to have high sugar concentrations (>23°Brix) which necessitate supplementation with nitrogen-containing nutrients to avoid problem alcoholic fermentations (Bisson & Butzke, 2000). Common industry practice has been to add diammonium phosphate (DAP) or proprietary blends containing DAP, amino acids, and/or other nutrients to grape musts (Munoz & Ingledew, 1990; Bell & Henschke, 2005). However, excessive addition of these formulations prior to alcoholic fermentation may yield enough residual nutrients after fermentation (*i.e.*, "carry-over") which support subsequent infections by spoilage microbes. Although Childs *et al.* (2015) reported that excessive nutrients added to synthetic grape juice media did not greatly impact growth of *Brettanomyces bruxellensis* in the resultant wines, little information is available regarding any impact of nutrient carry-over on bacteria such as pediococci. Furthermore, given the different nutritional requirements of *B. bruxellensis* and *Pediococcus* spp. or other lactic acid bacteria (Conterno *et al.*, 2006; Terrade & Mira de Orduña, 2009; Childs *et al.*, 2015; Von Cosmos & Edwards, 2016; Wade *et al.*, 2018), it cannot be assumed that residual nutrients in wine will affect these microorganisms in a similar manner.

The objective of this study therefore was to evaluate the impact of adding yeast nutrients to grape musts on the subsequent growth of *Pediococcus* spp. inoculated after alcoholic fermentation.

MATERIALS AND METHODS

Microorganisms and starter culture preparation

Microorganisms were propagated as nutrient-rich starter

*Corresponding author: E-mail address: edwardsc@wsu.edu
Acknowledgments: The authors gratefully acknowledge the Washington State Grape and Wine Research Program, Northwest Center for Small Fruits Research, regional and national wineries, and the School of Food Science at Washington State University for financial and material support

DOI: http://dx.doi.org/10.21548/40-1-3163

cultures prior to inoculation of grape musts or wines. *Saccharomyces cerevisiae* D254 was obtained as an active dry culture (Lallemand Inc., Montréal, Quebec, Canada) and maintained on yeast peptone dextrose (YPD) agar slants. Yeast starter cultures were prepared by aseptic transfer of single colonies into YPD broth (500 mL) with stirring (25°C). *Pediococcus parvulus* WS-7C and WS-29A (Edwards & Jensen, 1992), *P. parvulus* OW-1, *P. damnosus* OW-2, and *P. inopinatus* OW-8 (Strickland *et al.*, 2016), and *P. pentosaceus* ATCC 33316 (American Type Culture Collection, Manassas, VA, USA) were maintained on modified apple juice Rogosa medium (MR) agar slants as described by Fugelsang & Edwards (2007) or frozen in MR broth containing 30% v/v glycerol at -80°C. Bacterial starter cultures were prepared by transferring single colonies from MR agar to 100 mL of MR broth and incubating for one week (26°C). Weekly transfers of one mL of culture to fresh MR broth containing increasing amounts of ethanol (0%, v/v; 5%, v/v; 10%, v/v) were conducted over 21 days of incubation. Yeast and bacterial cells were harvested and suspended for must inoculation according to the protocol/ procedure of Osborne & Edwards (2006).

Synthetic grape juice media

Synthetic grape juice media (SGJM) which simulate grape musts as described by Wang *et al.* (2003) were prepared to yield three different concentrations of yeast assimilable nitrogen (YAN) from different proportions of ammonia and amino acids (Table 1). The low (55.2 mg N/L) and high (250 mg N/L) YAN media were prepared as described by Wang *et al.* (2003), the latter being identified as "medium YAN" in the present work. In addition, media with much higher amounts of YAN (530 mg N/L) and designated "high YAN" were prepared that contained Ala (413 mg/L), Arg (1.25 g/L), Asp (131 mg/L), Glu (522 mg/L), Gly (38 mg/L), His (283 mg/L), Ile (169 mg/L), Leu (205 mg/L), Lys (243 mg/L), Met (74 mg/L), Phe (169 mg/L), Pro (13.0 g/L), Ser (300 mg/L), Thr (261 mg/L), Trp (113 mg/L), Tyr (131 mg/L), and Val (1.14 g/L). Media were further supplemented with adenine sulfate (5 mg/L), biotin (10 µg/L), cytosine (5 mg/L), thymidine (5 mg/L), Tween™ 80 (0.05 g/L), uracil (5 mg/L), and xanthine (5 mg/L) obtained from Fisher Scientific (Pittsburgh, PA, USA), cysteine (5 mg/L) and calcium pantothenate (250 µg/L) obtained from Sigma-Aldrich (St. Louis, MO, USA), and guanine•HCl (5 mg/L) from Spectrum Chemical Mfg. Corp. (New Brunswick, NJ, USA). SGJM was sterile-filtered through 0.22 µm Steritop™ Sterile Vacuum Bottle-Top Filters (EMD Millipore, Billerica, MA, USA) into 3 L Celstir® vessels, in triplicate, prior to inoculation of *S. cerevisiae* at 10^4 cfu/mL. After 14 days of fermentation at 21°C, wines were again sterile-filtered into sterilized milk dilution bottles (100 mL). A powdered cellulose suspension (Sigmacell Type 20, Sigma Aldrich, St. Louis, MO, USA) was added to each bottle according to Childs *et al.* (2015). *Pediococcus* spp. were inoculated at initial populations of 10^4 to 10^5 cfu/mL (six replicates per treatment), the exception being *P. pentosaceus* which was inoculated at 10^3 cfu/mL. Wines were then incubated at 25°C for up to 75 days.

Spiking synthetic wines

After incubation with *Pediococcus* spp. for 75 days, the low and high YAN wines were subjected to additional nutrient supplementation and re-inoculation. Here, replicate wines were pooled, centrifuged (1000 x *g* for 10 min), and sterile-filtered (0.22 µm) with 10 mL dispensed into sterile 15 mm x 125 mm capped test tubes. All tubes received a sterilized cellulose suspension (1 g/L) and one of the following 0.22 µm filter-sterilized solutions: peptone (5 g/L), yeast extract (5 g/L), liver extract (1 g/L), Tween™ 80 (0.1 g/L), cysteine (0.2 g/L), asparagine (0.8 g/L), $MnSO_4$•$4H_2O$ (0.8 g/L), biotin (100 µg/L), or calcium pantothenate (1000 µg/L). *Pediococcus* spp. were re-inoculated, in triplicate, at initial populations of 10^5 to 10^6 cfu/mL, except *P. pentosaceus* which was inoculated at 10^3 cfu/mL. Tubes were incubated at 26°C for 24 days.

Cabernet Sauvignon musts

Cabernet Sauvignon grape juice concentrate (California Concentrate Company, Acampo, CA, USA) was diluted according to the manufacturer's instructions and sugar adjusted by adding equal proportions of glucose and fructose. In addition, the must was supplemented with biotin (10 µg/L), calcium pantothenate (250 µg/L), and other vitamins and trace minerals as described by Wang *et al.* (2003). Titratable acidity was adjusted to 6.8 g/L by the addition of tartaric and malic acids in a 5:3 (w/w) ratio while free SO_2 was removed to <3 mg/L using H_2O_2 as confirmed by the aeration oxidation method (Buechsenstein & Ough, 1978). After cold-settling overnight (4°C), the YAN content was adjusted with various amounts of DAP and/or amino acids (Wang *et al.*, 2003) as presented in Table 2.

For fermentation, musts were sterile-filtered through 0.45 µm Vitipore® II Plus Cartridge Filters (EMD Millipore, Billerica, MA, USA) into 3 L Celstir® fermentation vessels (Wheaton Science Products, Millville, NJ, USA), in triplicate. *S. cerevisiae* was inoculated at populations of 10^6 cfu/mL with fermentations conducted at 21°C for 14 days. Alcoholic fermentation was deemed complete when the concentrations of reducing sugars reached <2 g/L as determined by Bayer Clinitest® tablets (Fugelsang & Edwards, 2007). Each of the resulting wines were racked-off and subdivided into previously sterilized milk dilution bottles (100 mL). *Pediococcus* spp. were inoculated at 10^5 to 10^6 cfu/mL (six replicates per wine), the exception being *P. pentosaceus* which was inoculated at 10^4 to 10^5 cfu/mL prior to incubation at 25°C.

Analytical methods

Culturability was determined by spiral plating using an Autoplate 4000 (Spiral Biotech, Bethesda, MD, USA) on YPD for *Saccharomyces* and MR with cycloheximide (0.1 g/L) for *Pediococcus*. Plates were incubated at 26°C for three days (*Saccharomyces*) or 14 days (*Pediococcus* spp.) prior to enumeration. In addition, growth of *Pediococcus* spp. in test tubes was monitored by measuring absorbance at 550 nm using a FLUOstar® OPTIMA Microplate Reader (BMG Labtech, Cary, NC, USA) over a 24 day period. Here, population estimates were made by comparing optical density values to corresponding culturability (cfu/mL) on

TABLE 1
Composition of synthetic grape juice media pre- and post-alcoholic fermentation.

Pre-alcoholic fermentation	Low YAN	Medium YAN	High YAN
Alpha-amino nitrogen [mg/L]	29.6 ± 12	238 ± 5.8	524 ± 3.9
Ammonia [mg/L]	25.6 ± 0.92	12.2 ± 2.9	5.87 ± 0.64
°Brix	21.7 ± 0.50	22.5 ± 0.08	23.2 ± 0.14
pH	3.67 ± 0.01	3.62 ± 0.00	3.48 ± 0.00
Total YAN [mg N/L]	55.2 ± 2.0	250 ± 3.3	530 ± 3.3
Post-alcoholic fermentation			
Alpha-amino nitrogen [mg/L]	nd	35.0 ± 4.6	260 ± 8.1
Ammonia [mg/L]	nd	nd	nd
Ethanol [% ABV]	9.08 ± 0.11	13.0 ± 0.06	13.0 ± 0.09
Fructose [g/L]	41.2 ± 0.91	nd	0.167 ± 0.14
Glucose [g/L]	17.9 ± 2.5	nd	nd
pH	3.67 ± 0.01	3.81 ± 0.00	3.69 ± 0.00
Titratable acidity [g/L tartaric acid]	7.21 ± 0.16	6.58 ± 0.09	8.09 ± 0.05
Volatile acidity [g/L acetic acid]	0.44 ± 0.00	0.93 ± 0.01	0.88 ± 0.00

Values represent means of triplicate fermentations ± standard error
nd: Not detected

TABLE 2
Composition of Cabernet Sauvignon grape musts pre- and post-alcoholic fermentation.

Pre-alcoholic fermentation	Low YAN	Medium YAN	High YAN
Alpha-amino nitrogen [mg/L]	40.8 ± 0.92	196 ± 15	428 ± 17
Ammonia [mg/L]	26.0 ± 1.5	23.5 ± 0.63	9.80 ± 1.4
°Brix	19.3 ± 0.31	20.1 ± 0.03	20.7 ± 0.03
pH	3.90 ± 0.00	3.84 ± 0.00	3.83 ± 0.00
Total YAN [mg N/L]	66.9 ± 2.4	219 ± 15	438 ± 16
Post-alcoholic fermentation			
Alpha-amino nitrogen [mg/L]	2.5 ± 1.9	14.8 ± 2.0	165 ± 10
Ammonia [mg/L]	nd	1.26 ± 0.48	1.58 ± 0.27
Ethanol [% ABV]	10.4 ± 0.10	11.3 ± 0.03	11.4 ± 0.01
Fructose [g/L]	3.93 ± 1.2	nd	nd
Glucose [g/L]	nd	nd	nd
pH	3.72 ± 0.00	3.78 ± 0.00	3.89 ± 0.00
Titratable acidity [g/L tartaric acid]	6.64 ± 0.20	6.29 ± 0.04	6.38 ± 0.15
Volatile acidity [g/L acetic acid]	0.36 ± 0.00	0.32 ± 0.01	0.27 ± 0.02

Values represent means of triplicate fermentations ± standard error
nd: Not detected

standard growth curves prepared for each microorganism in MR broth.

Concentrations of ethanol were determined using an Agilent 1100 HPLC system equipped with refractive index detector (Agilent Technologies, Santa Clara, CA, USA) according to the method of Hall & Reuter (2007). Residual glucose and fructose were determined enzymatically using commercially available kits (Megazyme, K-FRUGL, Bray, Ireland). Titratable and volatile acidities were determined by standard methods (Edwards & Watson, 2013) while alpha-

amino nitrogen concentration was measured by the NOPA assay (Dukes & Butzke, 1998). Ammonia concentrations were determined using an ion selective electrode (McWilliam & Ough, 1974). Statistical analyses were performed with XLSTAT (Addinsoft, New York, NY, USA) with ANOVA and Fisher's LSD for mean separations.

RESULTS AND DISCUSSION

Fermentation of synthetic grape juice media

Determining the nutritional requirements of wine microorganisms often necessitates the utilization of synthetic media to accurately control concentrations of the nutrients in question (Liu *et al.*, 1995; Osborne & Edwards, 2006; 2007; Terrade & Mira de Orduña, 2009; Childs *et al.*, 2015). SGJM was therefore chosen to evaluate the impact of forms of nitrogen remaining after alcoholic fermentation on the growth of pediococci. This medium had been previously used to evaluate post-alcoholic growth of a spoilage yeast, *B. bruxellensis* (Childs *et al.*, 2015) and, with addition of purines and pyrimidines, *O. oeni* (Osborne & Edwards, 2006; 2007). Given similar nutrient requirements as *O. oeni*, enough nutrients should have been present to support *Pediococcus* spp. (Nakagawa & Kitahara, 1959).

Low, medium and high YAN synthetic grape musts yielded wines which contained different amounts of alpha-amino nitrogen and other chemical constituents (Table 1). Medium or high YAN grape musts produced dry wines (<2 g/L glucose and fructose) containing 13% (v/v) ethanol while those with low amounts of nitrogen did not complete fermentation. Low YAN musts yielded wines that only contained 9.08% (v/v) ethanol with approximately 59 g/L residual sugar (17.9 g/L glucose and 41.2 g/L fructose). The fact that these fermentations did not complete was not unexpected as the amount of alpha amino nitrogen in the musts was well below the minimum nitrogen requirements reported for successful fermentation (Agenbach, 1977; Spayd *et al.*, 1995). While residual alpha-amino nitrogen was not detected in low YAN wines, wines produced from medium and high YAN SGJM contained 35.0 and 260 mg N/L residual nitrogen, respectively (Table 1). Ammonia was not detected in any of the wines following alcoholic fermentation.

With the exception of *P. parvulus* (OW-1) and *P damnosus* (OW-2), bacterial culturability generally quickly declined after inoculation in the synthetic wines. Even with low amounts of ethanol present, populations of all strains quickly declined in low YAN wines, with many being reduced to undetectable levels within just a few days (Fig. 1). Strains WS-7C, WS-29A and OW-8 eventually recovered and became culturable but remained $\leq 10^4$ cfu/mL up to 50 days post inoculation in these wines. In contrast, populations of *P. parvulus* OW-1 and *P. damnosus* OW-2 decreased over a short time period (approximately 15 days) following inoculation before recovering to reach 10^5 cfu/mL in low YAN wines. Besides *P. parvulus* OW-1, none of the strains were recovered from wines produced from SGJM containing medium or high YAN (Fig. 1).

The limited growth of pediococci in wines produced from SGJM may be due to (a) synthesis of an inhibitory substance(s) by *S. cerevisiae* during the alcoholic fermentation or (b) absence or insufficient quantity of an unidentified required nutrient. For example, better growth in wines produced from SGJM with low YAN compared to medium and high amounts may have been due to the lower concentration of ethanol (9% vs. 13% v/v). While ethanol tolerances for some pediococci have been reported to be upwards of 14% to 16% (Maret & Sozzi, 1977; Silver & Leighton, 1981; Edwards & Jensen, 1992), Davis *et al.* (1988) noted that growth of pediococci decreased as ethanol increased from 12.5% to 15% v/v. In fact, pediococci exhibited growth in low YAN wines despite limited concentrations of nitrogen-containing substances compared to medium and high YAN wines that contained measurable amounts of alpha amino nitrogen (35.0 and 260.1 mg N/L, respectively).

Aside from ethanol, *S. cerevisiae* is known to produce a range anti-bacterial compounds (Edwards & Beelman, 1987; Capucho & San Ramao, 1994; Carrete *et al.*, 2002; Comitini *et al.*, 2005; Osborne & Edwards, 2006; 2007). While Osborne & Edwards (2006) reported increased inhibition of *O. oeni* when grown in wines fermented from musts containing high amounts of nitrogen, Wibowo *et al.* (1988) observed growth of *P. parvulus* in Shiraz wines following alcoholic fermentation and MLF by *Leuconostoc oenos* (*O. oeni*). In fact, Vilanova *et al.* (2007) noted a correlation between nitrogen concentration and production of decanoic acid by wine yeast, a compound associated with the inhibition of *O. oeni* (Edwards & Beelman, 1987).

Rather than explore the possible existence of yeast-produced inhibitory compounds, poor growth in the synthetic wines was investigated through addition of selected nutrients. Additions of asparagine, biotin, calcium pantothenate, cysteine, liver extract, manganese sulfate, or Tween 80™ to wines made from SGJM had little to no effect on growth for most strains (data not shown) despite being important growth factors for lactic acid bacteria (Nakagawa & Kitahara, 1959; De Man *et al.*, 1960; Garvie & Gregory, 1961; Holzapfel *et al.*, 2009). However, addition of peptone or yeast extract improved growth of strain WS-7C (Fig. 2), thereby suggesting a lack of nutritional component(s) as opposed to the presence of inhibitory substance(s). In fact, peptone encouraged growth of five of the six strains studied (excluding *P. pentosaceus*) while yeast extract promoted strong growth of all six strains (data not shown). These results were in agreement with Nel *et al.* (2001) which demonstrated better growth of *P. damnosus* in MRS media if supplemented with peptone or meat extract, with yeast extract or tryptone also improving growth to a lesser extent.

The specific cause of growth stimulation due to peptone and/or yeast extract is not well understood. Early research by Snell *et al.* (1937a; 1937b) reported that the stimulatory impact of peptone on lactic acid bacteria may be due to a basic amino acid or a compound with similar properties. Being prepared from cytoplasmic constituents of *S. cerevisiae* (Ángeles Pozo-Bayón *et al.*, 2009), yeast extract is rich in peptides (Martínez-Rodriguez *et al.*, 2001) and contributes free amino acids, lipids, vitamins/minerals, and several trace elements (Grant & Pramer, 1962; Ángeles Pozo-Bayón *et al.*, 2009). While it is difficult to identify specific factor(s) responsible for the growth enhancement of pediococci, commercial musts or wines are

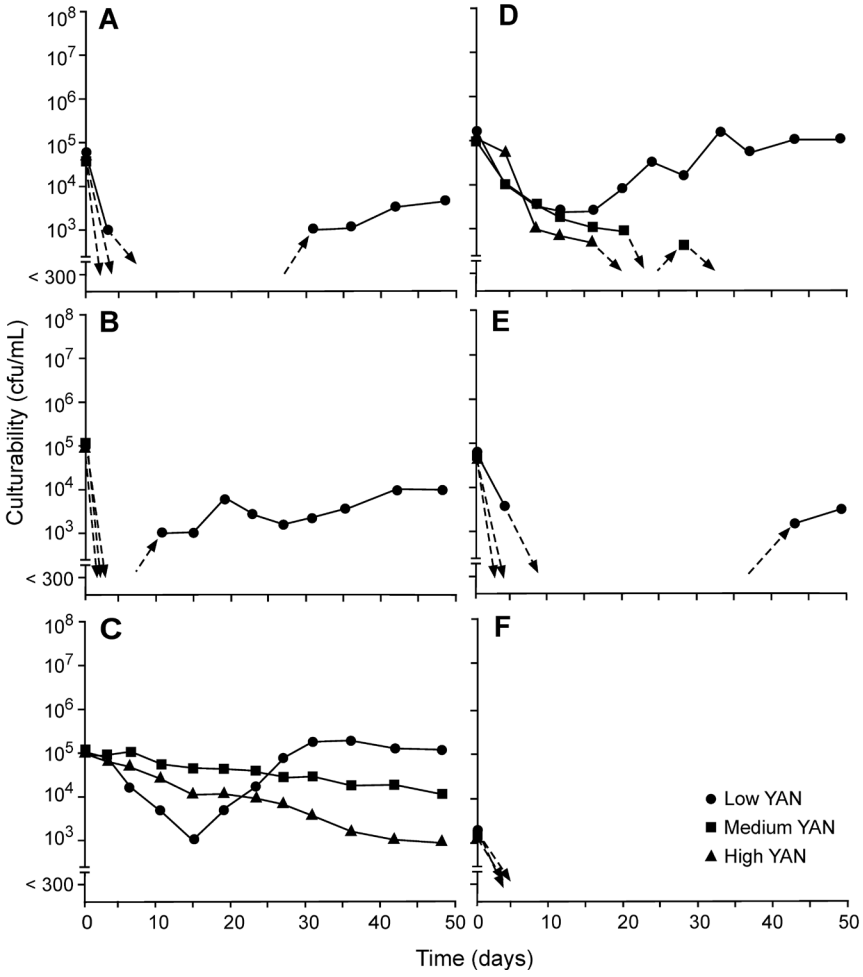

FIGURE 1

Culturability of *P. parvulus* strains WS-7C (A), WS-29A (B), and OW-1 (C), *P. damnosus* OW-2 (D), *P. inopinatus* OW-8 (E), or *P. pentosaceus* ATCC 33316 (F) in synthetic wines prepared from SGJM initially containing 55.2 mg N/L (low), 250 mg N/L (medium), or 530 mg N/L (high) YAN before alcoholic fermentation.

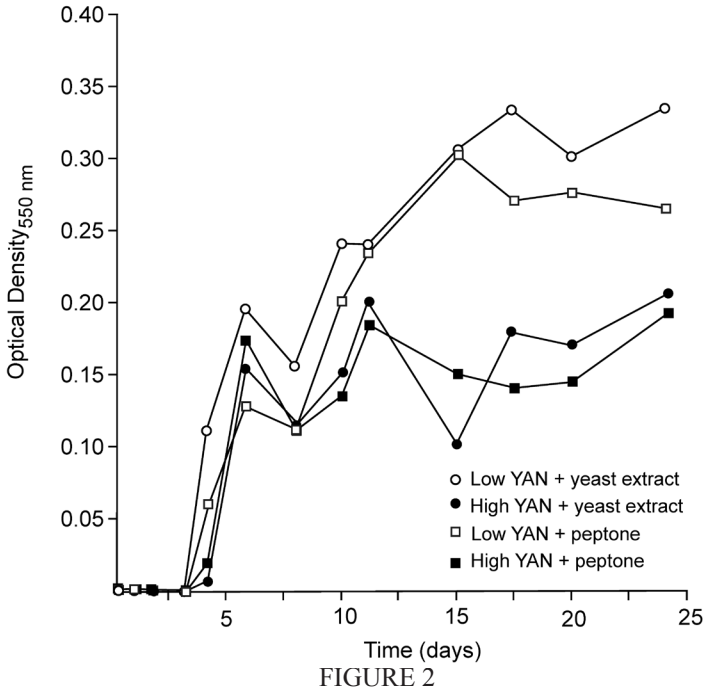

FIGURE 2

Re-inoculation and growth of *P. parvulus* WS-7C in synthetic wines additionally supplemented with yeast extract or peptone.

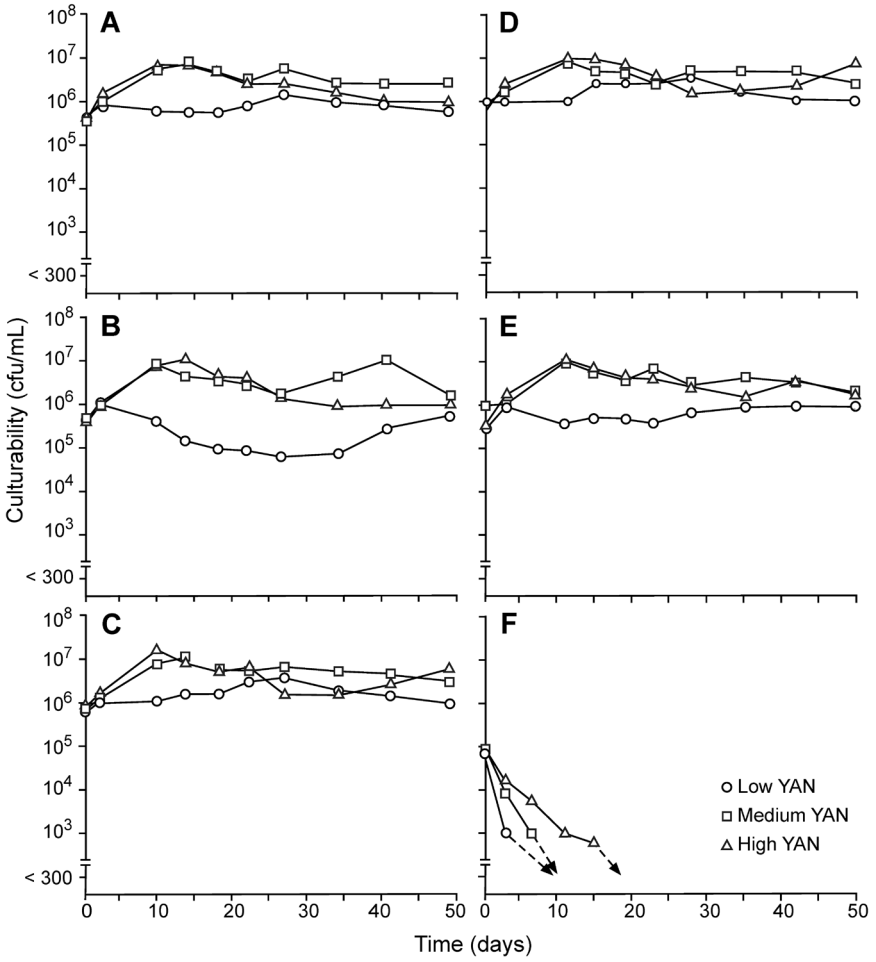

FIGURE 3
Culturability of *P. parvulus* strains WS-7C (A), WS-29A (B), and OW-1 (C), *P. damnosus* OW-2 (D), *P. inopinatus* OW-8 (E), or *P. pentosaceus* ATCC 33316 (F) in Cabernet Sauvignon wines prepared from grape musts initially containing 67 mg N/L (low), 219 mg N/L (medium), or 438 mg N/L (high) YAN before alcoholic fermentation.

often supplemented with inactive dry yeast preparations as a means to stimulate alcoholic or malolactic fermentations (Ángeles Pozo-Bayón *et al.*, 2009). Furthermore, wines can be stored with lees during aging (Ángeles Pozo-Bayón *et al.*, 2009) where enzymatic degradation of yeast cells liberates nitrogen-containing compounds (peptides and free amino acids), polysaccharides, and fatty acids (Charpentier & Feuillat, 1993).

Fermentation of Cabernet Sauvignon musts

Given poor growth in wines made from SGJM, pediococci were inoculated into Cabernet Sauvignon wines where YAN values were altered prior to alcoholic fermentation. Alcoholic fermentation conducted by *S. cerevisiae* was complete (<2 g/L residual sugar) within ten days for all treatments with the exception of the low YAN must which became stuck with an average of 3.9 g/L residual fructose (Table 2). Wines produced from musts containing low or medium amounts of YAN also contained lower concentrations of residual amino nitrogen than those made from high YAN musts, in agreement with Sturgeon *et al.* (2013) and Childs *et al.* (2015). Ammonium was depleted from all wines to concentrations <2 mg N/L.

Unlike the observations using SGJM, most of the *Pediococcus* species grew well in the Cabernet Sauvignon wines. However, growth of pediococci in wines produced from low YAN musts differed from wines produced from medium or high YAN (Fig. 3). Specifically, populations of $\geq 10^7$ cfu/mL were achieved by *P. parvulus* (WS-7C, WS-29A and OW-1), *P. damnosus* (OW-2), or *P. inopinatus* (OW-8) in wines produced from medium or high YAN grape musts, the exception being *P. pentosaceus* (ATCC 33316) which died-off soon after inoculation. Although less growth was observed in wines fermented from low YAN musts, bacterial populations of 10^5 to 10^6 cfu/mL were reached despite containing only 2.5 mg N/L. In fact, wines prepared from medium YAN musts contained nearly 11 times less residual amino nitrogen than high YAN (Table 2) yet were able to support similar populations of pediococci. These observations suggest that low concentrations of residual amino nitrogen were enough to support the growth of different species of *Pediococcus*, similar to previous findings regarding *Brettanomyces bruxellensis* (Childs *et al.*, 2015).

Although stuck/sluggish fermentations may be more susceptible to unwanted microbial growth (Bisson & Butzke, 2000), wines produced from the medium and high

YAN grape musts that completed fermentation similarly supported bacterial growth as those produced from low YAN grape musts that did not complete fermentation. In this case, the presence of higher residual sugar and lower ethanol in the low YAN wines did not improve pediococci growth compared to the medium and high YAN wines, although all wines contained <12% v/v alcohol.

The frequency at which winemakers encounter nitrogen deficient grape musts has resulted in supplementation guidelines to avoid the addition of excessive amounts of nutrients (Ingledew & Kunkee, 1985; Bisson & Butzke, 2000) in order to avoid risk of subsequent microbial spoilage (Beltran *et al.*, 2004). However, growth of pediococci in the present study were not greatly impacted by increased levels of residual nitrogen in the wines. While negligible levels of residual amino nitrogen present in some of the Cabernet Sauvignon wines led to increased lag times for bacterial growth, these wines ultimately reached similar populations as wines containing high concentrations of residual nitrogen. As such, the results of this study suggest that the amount of ethanol had more of an inhibitory impact on *Pediococcus* spp. growth than the presence of amino nitrogen. Additional research involving *O. oeni*, *Lactobacillus* spp., and/or non-*Saccharomyces* yeasts is warranted to better evaluate impacts of excessive must supplementation on the potential for subsequent spoilage by microorganisms other than *Pediococcus* spp.

CONCLUSIONS

Increased YAN concentration in grape musts resulted in increased residual amino nitrogen in subsequent wines which theoretically could be utilized by *Pediococcus* spp. after completion of fermentation. However, amino nitrogen had a limited effect on *Pediococcus* growth in comparison to ethanol concentration, the latter of which impacted growth more than nutrient availability. The poor growth of *Pediococcus* spp. in synthetic wines was improved with addition of peptone or yeast extract, suggesting the absence or insufficient concentration of an unidentified essential growth factor(s).

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