YEAR 3 PROGRESS REPORT – January 2021

I. PROJECT TITLE:

Utilizing malolactic fermentation as a tool to prevent *Brettanomyces bruxellensis* wine spoilage.

II. PRINCIPAL INVESTIGATOR:

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III. SUMMARY:

Brettanomyces bruxellensis is considered one of the most problematic wine spoilage yeasts due to the difficulty of controlling it, the potential significant financial losses due to loss of wine quality, and the cost of prevention and remediation measures. Wine is particularly vulnerable to *B. bruxellensis* infection during and shortly after the malolactic fermentation (MLF) as SO_2 cannot be added until this process is complete. It has been suggested that conducting a rapid MLF initiated by inoculation of *Oenococcus oeni* is a useful strategy to prevent *B. bruxellensis* spoilage as this minimizes the length of time the wine is not protected by SO_2. This project investigates an additional benefit of conducting a rapid MLF, the prevention of *B. bruxellensis* growth due to inhibitory interactions with *O. oeni*. Pinot noir wine (no SO_2 additions, no MLF) was produced and used to test the ability of a large number of commercial *O. oeni* strains to inhibit *B. bruxellensis* growth at the end of MLF. Sterile filtered wine was inoculated with one of eleven commercial *O. oeni* strains and growth and malic acid monitored. When MLF was complete, wines were inoculated with a select strain of *B. bruxellensis* and growth and volatile phenol production monitored.

All *O. oeni* strains tested inhibited the growth of *B. bruxellensis* UCD2049 in Pinot noir wine with *O. oeni* strain variation observed. *O. oeni* strains Alpha, 350, VP41, MBR31 and PN4 most strongly inhibited growth of *B. bruxellensis* UCD2049, while strains CH11, Omega, Beta, and VFO 2.0 inhibited *B. bruxellensis* to a lesser extent. The potential mechanism of this inhibition was investigated by using a dialysis membrane to physically separate *O. oeni* and *B. bruxellensis* cells but allow free movement of nutrients and other potential inhibitory compounds. The physical separation of *O. oeni* from *B. bruxellensis* relieved the inhibition of *B. bruxellensis* by *O. oeni* that occurred when the two microorganism were in present together. These results indicate that inhibition is not due to nutrient depletion by *O. oeni* as nutrients could flow freely across the dialysis membrane. It is also unlikely that *B. bruxellensis* inhibition was due to the production of an inhibitory compound by *O. oeni* as any potential inhibitory compound would also have passed through the dialysis membrane. Instead, these results provide strong evidence that the inhibition of *B. bruxellensis* by *O. oeni* is due to cell-cell contact.

The sensitivity of additional *B. bruxellensis* strains to *O. oeni* was also determined. While *B. bruxellensis* UCD2049 populations declined rapidly when inoculated into Pinot noir wine that had just completed MLF with *O. oeni* Alpha, growth of the other *B. bruxellensis* strains tested was not impacted. Why *B. bruxellensis* strain UCD2049 was inhibited by *O. oeni* while the other *B. bruxellensis* strains were not was subsequently investigated. Initial experiments considered whether ethanol tolerance between *B. bruxellensis* strains impacted inhibition by *O. oeni*. Given that earlier experiments had been conducted in 13% (v/v) wine, wines were instead adjusted to 12.5% or 14% (v/v) ethanol. In low (12.5%) ethanol wine that had undergone MLF, *B. bruxellensis* UCD2049 grew well, in contrast to what was observed in 13% wine where growth was inhibited. *B. bruxellensis* strains AWRI-1499 and Copper Mountain also grew well in low ethanol wine with no difference between treatments. In higher ethanol wine, *B. bruxellensis* UCD2049 struggled to grow whether the wine had undergone MLF or not. In contrast, *B. bruxellensis* strains AWRI-1499 and Cooper Mountain grew well in the higher ethanol wine. *B. bruxellensis* strains AWRI-1499 populations recovered slower in wine that had undergone MLF while the opposite occurred for strain Copper Mountain. These results demonstrate that ethanol tolerance differences between *B. bruxellensis* strains impact their inhibition by *O. oeni*. For example, strain UCD2049 was not inhibited by *O. oeni* in wine at 12.5% ethanol but was inhibited in 13% and 14% ethanol wine. Additional experiments will be conducted where pH will also be considered as tolerance to this factor is known to differ between *B. bruxellensis* strains. Experiments are also underway exploring how long MLF induced *B. bruxellensis* inhibition last as well as whether *B. bruxellensis* inhibition occurs if infection happens at the beginning or mid-point of MLF.

IV. OBJECTIVES AND EXPERIMENTS CONDUCTED TO MEET STATED OBJECTIVES:

Objective 1. Investigate *Oenococcus oeni* inhibition of *B. bruxellensis* growth and volatile phenol production and determine if strain variability exists

A large number of commercial *O. oeni* strains were collected and pure cultures of each strain obtained by streaking for single colonies and growing a single colony in broth before storing culture at -80°C in glycerol. *B. bruxellensis* strains were collected from the UC Davis culture collection, the AWRI culture collection, the WSU *Brettanomyces* culture collection (Dr. Charles Edwards) and various culture collections at OSU (Bakalinsky and Curtin). As with *O. oeni, B. bruxellensis* cultures were streaked for single colonies, grown in broth, and stored at -80°C in glycerol.

To study interactions between *O. oeni* and *B. bruxellensis,* Pinot noir wine was produced in 2018, 2019, and 2020 where no SO_2 additions were made and where MLF was prevented by sterile filtration at the completion of alcoholic fermentation. Pinot noir grapes from the Oregon State University vineyard were destemmed, an addition of Fermaid K (0.25 g/L) was made followed by inoculation with *Saccharomyces cerevisiae* RC212. Fermentations were conducted at 27 °C with twice-daily punch downs and °Brix and temperature monitored daily. At the completion of alcoholic fermentation (< 0.5 g/L reducing sugar) wines were pressed, cold settled, and filtered through 5 µm and 1 µm cartridge filters. Wine was then sterile filtered (0.45 µm PES

membrane filter) and stored at 4°C until needed for experiments. Basic wine composition (pH, TA, alcohol, malic acid) was measured using standard methods. Wine hydoxycinnamic acids (volatile phenol pre-cursors) were measured by HPLC-DAD (Chescheir et al. 2015). The basic chemical composition of the Pinot wines produced is listed in Table 1.

Oenococcus oeni screening

Pinot noir wine produced in 2018 was used to investigate the impact of a number of commercially available *O. oeni* strains on the growth and volatile phenol production of *B. bruxellensis*. The 2018 Pinot noir wine was adjusted to 13% (v/v) ethanol by the addition of sterile water. Eleven commercial *O. oeni* strains (VP41, Beta, Alpha, PN4, CH-11, CH-16, CH-35, MBR31, VFO 2.0, 350 PreAc, Omega) were streaked for single colonies on MR plates and a single colony was inoculated into broth. After 10 days incubation, *O. oeni* were harvested by centrifugation and inoculated into sterile filtered wine at approx. 10^6 CFU/mL. *O. oeni* populations are monitored every five days by plating on MRS media while MLF was monitored by enzymatic analysis of malic acid. When MLF was complete, wines were inoculated with *B. bruxellensis* UCD2049 at approx. 10^4 CFU/mL. A set of wines that did not undergo MLF (control) was also inoculated with *B. bruxellensis* UCD2049. All treatments were conducted in triplicate. *B. bruxellensis* populations were monitored by plating on YPD media for a minimum of 60 days.

Brettanomyces bruxellensis screening

Pinot noir wine produced in 2019 was used to test the sensitivity of a number of *B. bruxellensis* strains to *O. oeni*. A single *O. oeni* strain, Alpha, was chosen to test against the *Brettanomyces* strains based on results from the *O. oeni* screening experiments. Sterile filtered 2019 Pinot noir wine was ethanol adjusted to 13% by the addition of sterile water and divided into 100 ml aliquots in 100 mL media bottles and inoculated with *O. oeni* Alpha at approx. 10^6 CFU/mL. MLF was monitored by enzymatic analysis of malic acid and considered complete when malic acid was < 0.05 g/L. *O. oeni* populations were determined at the end of MLF by plating on MRS media. *B. bruxellensis* strains were prepared from pure cultures as previously described and inoculated at approx. 10^4 CFU/mL. Pinot noir wines that had not undergone MLF was also inoculated with the various *B. bruxellensis* strains. All treatments were conducted in triplicate and *B. bruxellensis* populations were monitored by plating on YPD media. At the completion of the experiment, wine samples were assessed for volatile phenols by GC-MS as described by Zhou et al. (2015).

Interactions between ethanol and presence of O. oeni

An experiment in 2019 Pinot noir wine investigated if the differences in *B. bruxellensis* susceptibility to *O. oeni* is related to their sensitivity to ethanol. Inhibitory conditions in a wine provide a combined effect on the ability of *B. bruxellensis* to grow and this experiment will determine if the presence of *O. oeni* is an additional "hurdle" that *Brettanomyces* needs to overcome in wine. AN overview of the experimental design is shown in Figure 1. MLF was induced in the Pinot noir by addition of *O. oeni* Alpha. At the completion of MLF, wines were adjusted to 12.5% or 14% ethanol by the addition of Everclear or sterile water. A set of wines that did not undergo MLF was also adjusted to 12.5% or 14% ethanol by the addition of Everclear or sterile water. The pH of the non-MLF wines was also adjusted to the same pH as the wines that had undergone MLF to ensure that pH differences between the wines did not interfere

with the experiment. After ethanol adjustments, all wines were inoculated with either *B. bruxellensis* strain UCD2049, strain AWRI-1499, or strain Copper Mountain at approx. 10^3 CFU/mL. All treatments were conducted in triplicate. *B. bruxellensis* populations were monitored by plating on YPD media for a minimum of 60 days.

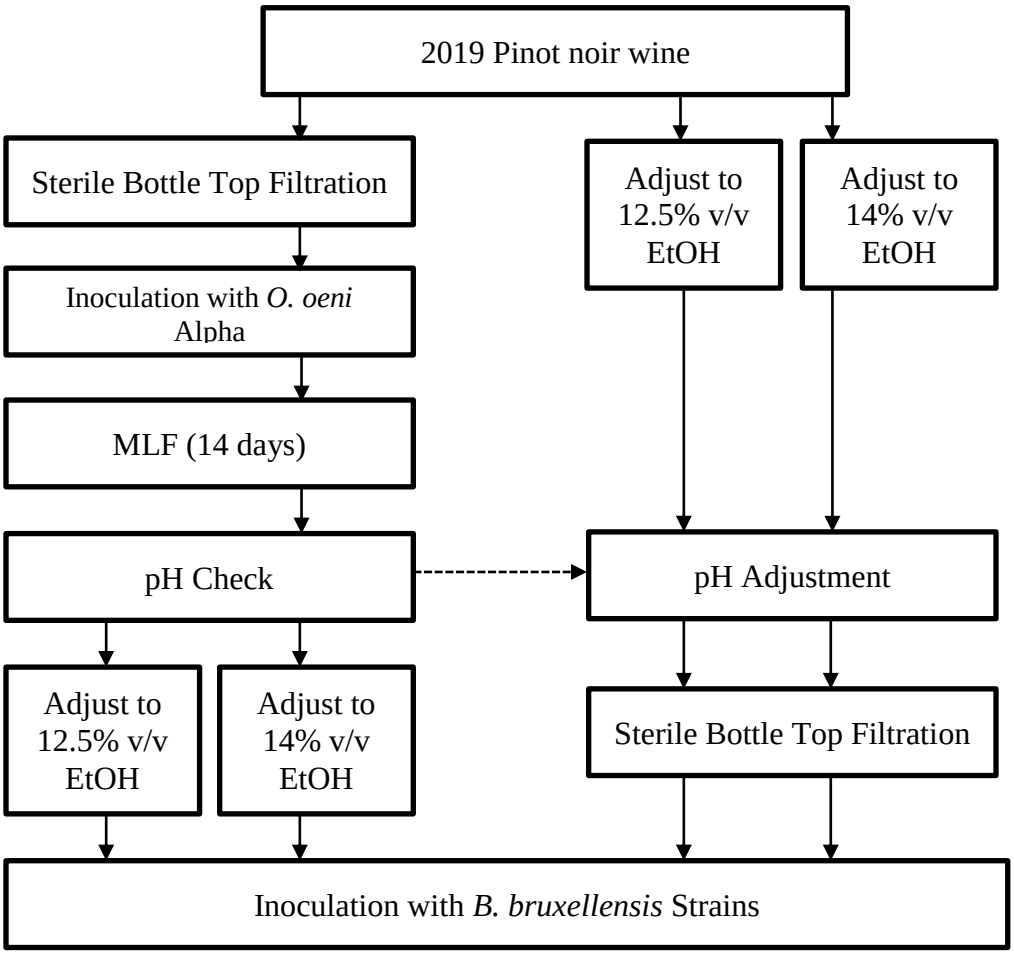

Figure 1. Experimental design for impact of *O. oeni* strain Alpha on a *B. bruxellensis* strains in high and low ethanol wine

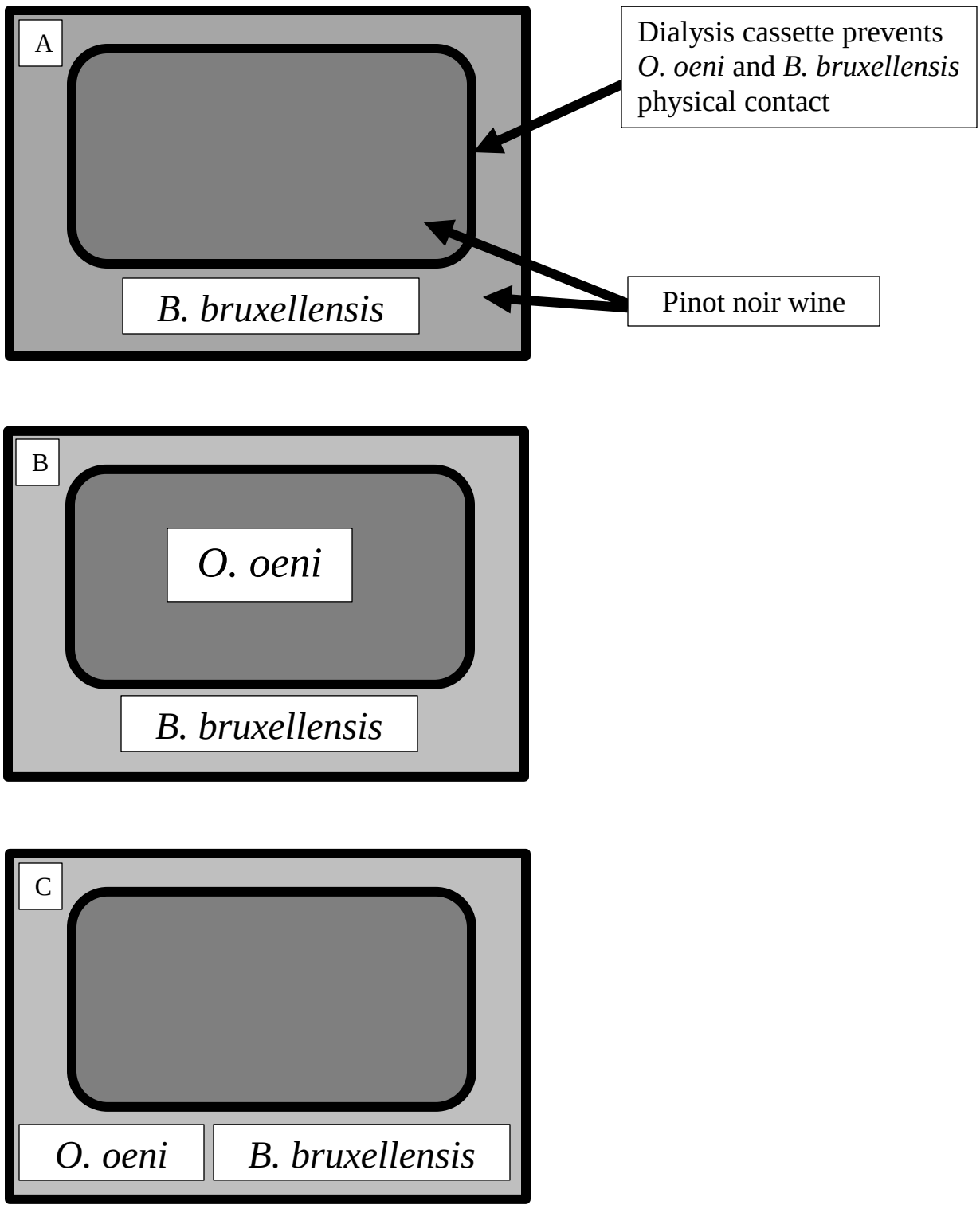

Figure 2. Experimental set-up for dialysis cassette experiment. Treatment (A) assess growth of *B. bruxellensis* UCD-2049 by itself in wine that has not undergone MLF. Treatment (B) assess growth of *B. bruxellensis* UCD-2049 in wine that has undergone MLF with *O. oeni* Alpha where *O. oeni* and *B. bruxellensis* were physically kept separate by dialysis membrane. Treatment (C) assess growth of *B. bruxellensis* UCD-2049 in wine that has undergone MLF with *O. oeni* Alpha where *O. oeni* and *B. bruxellensis* are in physical contact.

Objective 2. Determine mechanism by which *B. bruxellensis* is inhibited by *O. oeni*
Experiments to determine the mechanism by which *B. bruxellensis* is inhibited by *O. oeni* were undertaken. An experimental set-up utilizing dialysis membrane cassettes (10,000 Dalton molecular weight cut-off) sourced from Thermo-Scientific was used. The set-up of the treatments are outlined in Figure 2. To remove contaminants such as sulfur dioxide from the dialysis membranes, each cassette was passed through a washing procedure. Dialysis cassettes were prepared by aseptically soaking in: 1% EDTA, 0.3% sodium sulfite, and 0.2% sulfuric acid. Cassettes were soaked in sterile deionized (DI) water between each rinse step. Cassettes were then soaked in 0.3% hydrogen peroxide for ten minutes to remove SO_2, soaked in DI water o/n, and finally filled with sterile filtered 2019 Pinot noir wine. After 24hrs, the wine was tested for free and bound SO_2 to ensure that the washing steps had been successful. *O. oeni* Alpha was inoculated into the cassettes at approx. 10^6 CFU/mL as indicated in Figure 1. MLF was monitored by enzymatic assay and at the end of MLF *B. bruxellensis* UCD-2049 was inoculated into all treatments at approx. 10^5 CFU/mL. All treatments were conducted in triplicate and *B. bruxellensis* populations were monitored by periodically plating on YPD agar.

Objective 3. Investigate impact of timing of *B. bruxellensis* infection relative to malolactic fermentation on growth inhibition and persistence of inhibition
Results to date have shown that *B. bruxellensis* is inhibited when inoculated into wine at the end of MLF. An xperiments was conducted to determine if this inhibition also occurs if *B. bruxellensis* infection occurs at the beginning or during MLF. 2019 sterile filtered Pinot noir wine was used for the following treatments:

1) Control (no-MLF, no microorganisms)
2) Inoculate wine with only *O. oeni* Alpha at 10^6 CFU/mL
3) Inoculate wine with only *B. bruxellensis* UCD-2049 at 10^3 CFU/mL
4) Inoculate wine with both *O. oeni* Alpha (10^6 CFU/mL) and *B. bruxellensis* UCD-2049 (10^3 CFU/mL)
5) Inoculate wine undergoing MLF with *O. oeni* Alpha with *B. bruxellensis* (10^3 CFU/mL) part way through MLF (target 50% malic reduction)
6) Inoculate wine that has completed MLF with *B. bruxellensis* UCD-2049

The initial experiment was meant to run for 90 days but due to COVID-19 restrictions at the University, the experiment was unable to be completed. A partial set of results were generated and are included in this report. This experiment will be repeated using wine produced in 2020.

V. <u>SUMMARY OF MAJOR RESEARCH ACCOMPLISHMENTS AND RESULTS:</u>

A number of *B. bruxellensis* strains were collected from various culture collections including strains isolated from Oregon wineries. The genetic and physiological characteristics of many of these strains has previously been determined (Conterno et al 2006; Jensen et al. 2009; Curtin et al. 2012; Oswald and Edwards 2017) and this information was used to help determine which strains to tested for their sensitivity to *O. oeni*. For example, strain AWRI-1499 is reported to have high tolerance to SO_2 (Curtin et al. 2012), while strain UCD-2049 has low tolerance to SO_2 (Conterno et al 2006; Jensen et al. 2009; Curtin et al. 2012). Additionally, strain WSU-i1a is more ethanol tolerant than strain WSU-F3 (Oswald and Edwards 2017).

Table 1. Pinot noir wine parameters

	pH	TA (g/100 mL)	Alcohol % (v/v)	Malic acid (g/L)	*p*-coumaric acid (mg/L)
2018 Pinot noir	3.45	0.64	14.5	1.56	1.1
2019 Pinot noir	3.48	0.59	13.7	1.29	1.2
2020 Pinot noir	3.39	0.68	13.9	1.85	1.0

Table 2. *O. oeni* strains used for screening

Strain	**Source**
Alpha	Lallemand
Beta	Lallemand
VP41	Lallemand
MBR31	Lallemand
CH35	Chr. Hansen
CH16	Chr. Hansen
CH11	Chr. Hansen
VFO 2.0	Chr. Hansen
PN4	Lallemand
350 PreAc	Laffort
Omega	Lallemand

Table 3. *B. bruxellensis* strains used for screening

Strain	Source	Collection
UCD-2049	Wine, Pinot noir, New Zealand	UC Davis culture collection
AWRI-1499	Wine, Australia	AWRI culture collection
Y18	Wine, Australia	Curtin OSU culture collection
Y16	Wine, Australia	Curtin OSU culture collection
WSU-F3	Wine, Cabernet Sauvignon, WA	Edwards WSU culture collection
WSU-i1a	Wine, Syrah, WA	Edwards WSU culture collection
UCD-738	Wine, MO	UC Davis Culture Collection
Copper Mountain	Wine, Pinot noir, OR	Bakalinsky culture collection

<u>*O. oeni* screening results:</u>

B. bruxellensis UCD2049 was inoculated into 2018 Pinot noir wine (ethanol adjusted to 13% v/v) that had not undergone MLF or had just completed MLF using one of eleven commercial strains of *O. oeni*. When inoculated into wine that had not undergone MLF *B. bruxellensis* grew well with no decline in culturable cells. *B. bruxellensis* populations reached above 10^6 CFU/mL 14 days after inoculation into the wine. In contrast, *B. bruxellensis* culturable cell populations declined rapidly when inoculated into wine that had just completed MLF no matter which *O. oeni* had conducted MLF (Figure 2-4). Variation in *B. bruxellensis* growth was observed depending on which *O. oeni* strain conducted MLF. In the case of wines that underwent MLF with *O. oeni* strain 350, Alpha, PN4, VP41, and MBR31, *B. bruxellensis* culturable cells were not detected again during the course of the experiment (49 days). In wines where MLF was conducted by *O. oeni* strain CH35 or CH16, low populations of *B. bruxellensis* were detected during the duration of the experiment. In wines where MLF was conducted by either *O. oeni*

strain Beta, Omega, CH11, or VFO 2.0, *B. bruxellensis* populations began to revover by the end of the experiment. For example, in wine where MLF was conducted by *O. oeni* CH11, *B. bruxellensis* populations rose from an undetectable level 14 days after inoculation to approx. 10^4 CFU/mL by day 49 (Figure 3). However, even in the case of CH11, *B. bruxellensis* populations were reduced to very low populations for 2-3 weeks.

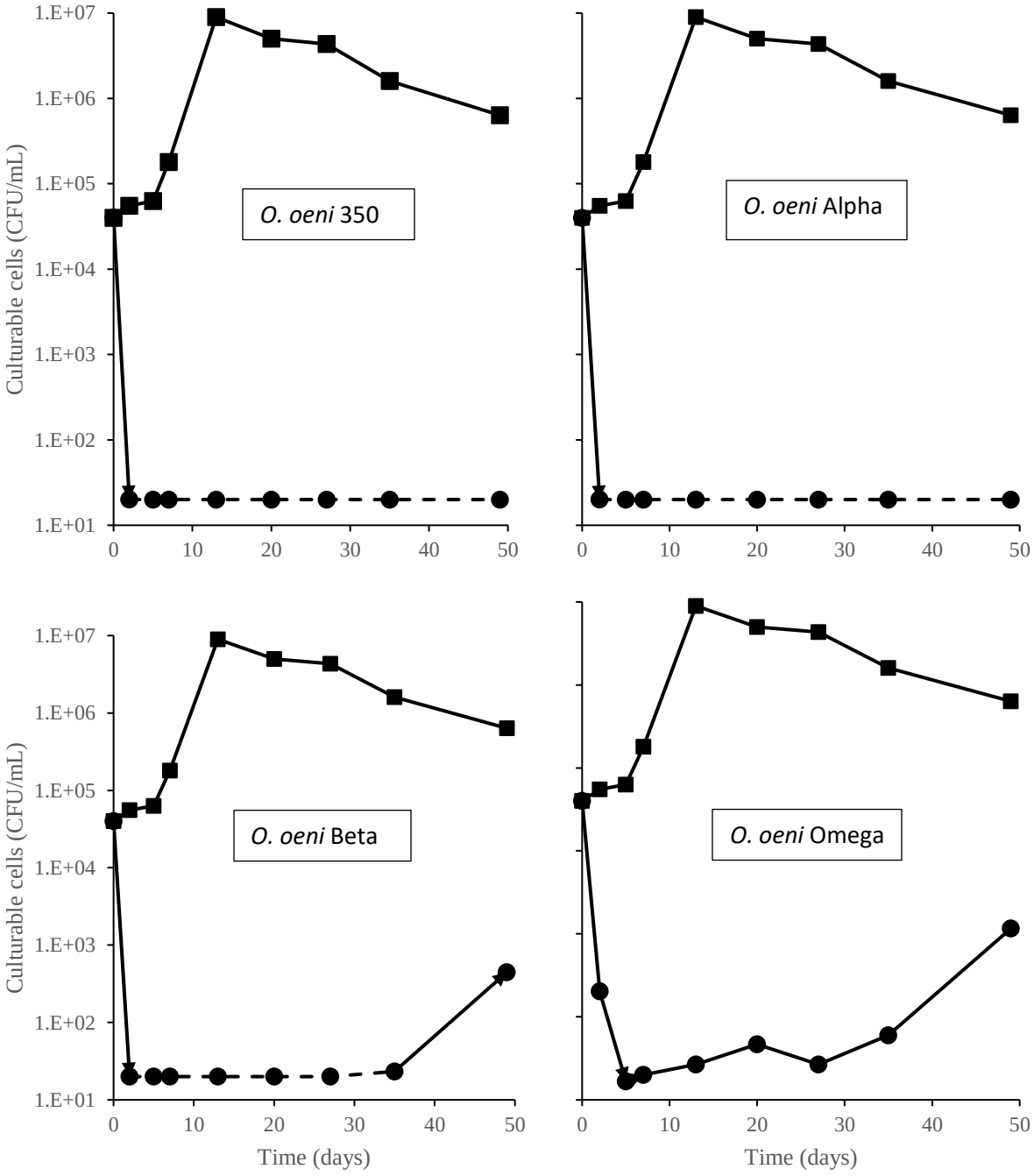

Figure 3. Growth of *Brettanomyces bruxellensis* UCD2049 after inoculation into Pinot noir wine that had not undergone MLF (■) or had undergone MLF with various *O. oeni* strains (●).

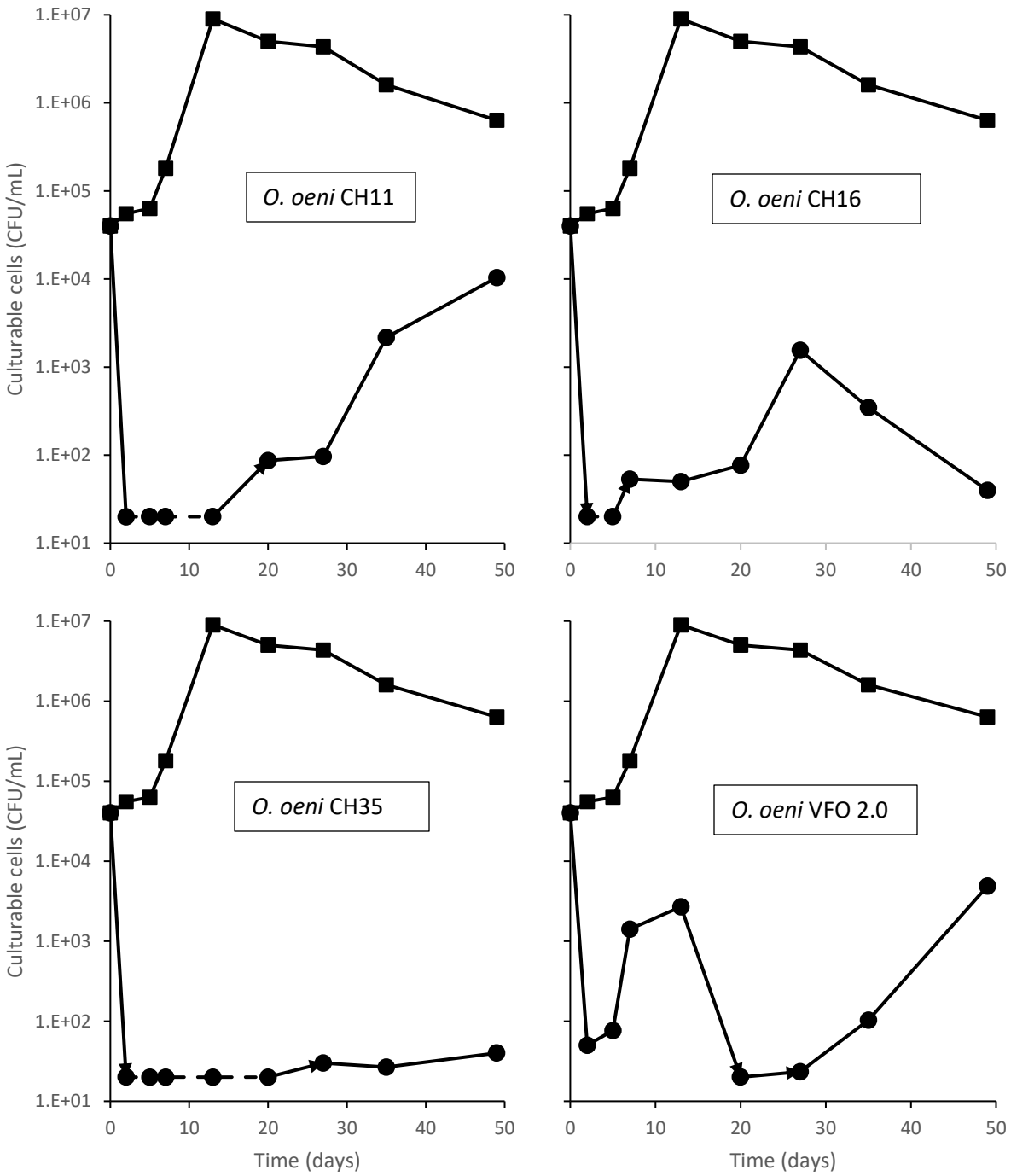

Figure 4. Growth of *Brettanomyces bruxellensis* UCD2049 after inoculation into Pinot noir wine that had not undergone MLF (■) or had undergone MLF with various *O. oeni* strains (●).

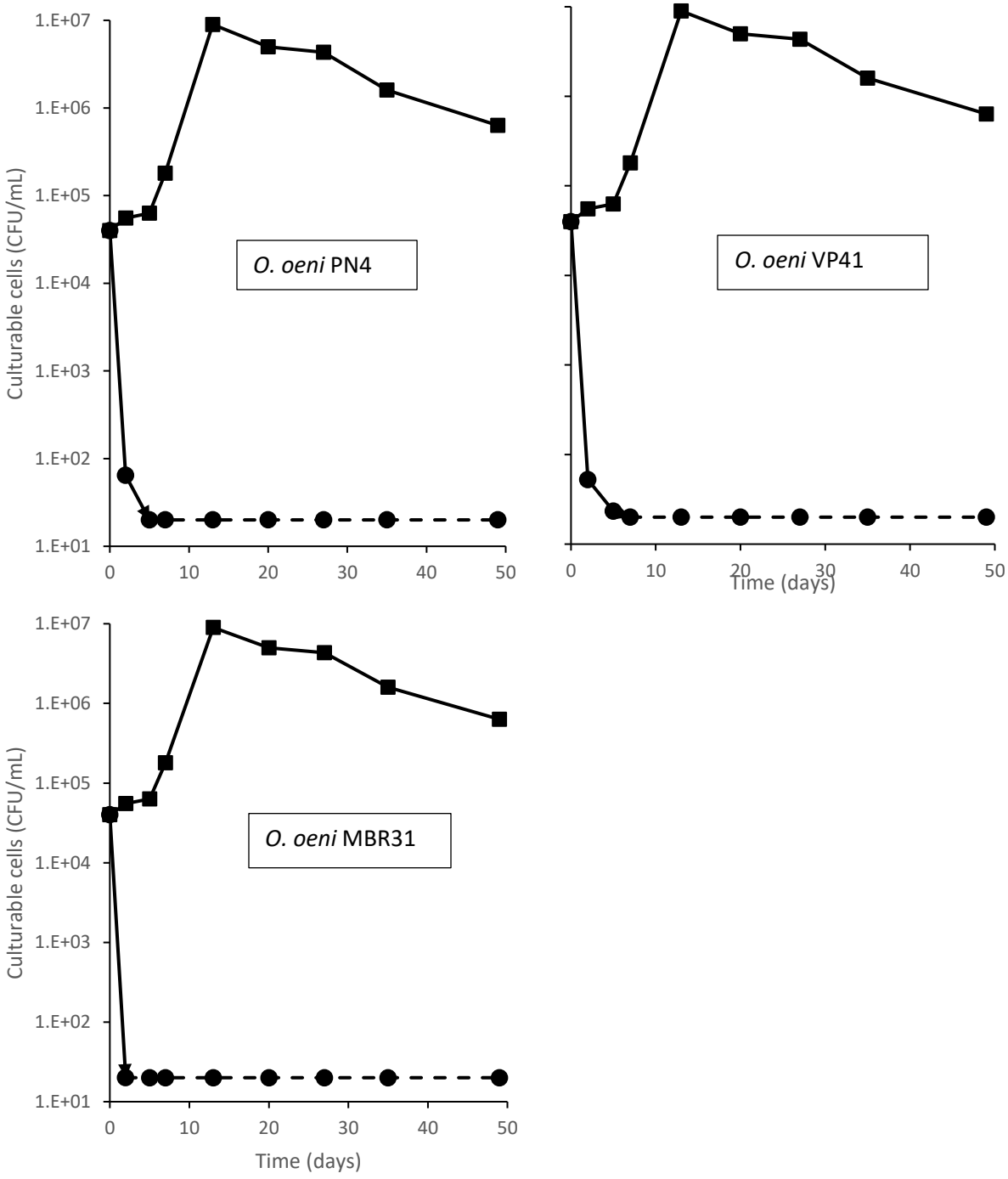

Figure 5. Growth of *Brettanomyces bruxellensis* UCD2049 after inoculation into Pinot noir wine that had not undergone MLF (■) or had undergone MLF with various *O. oeni* strains (●).

O. oeni mechanism of inhibition

To investigate the mechanism of inhibition resulting from MLF, *B. bruxellensis* strain UCD-2049 was inoculated into wine that had or had not undergone MLF using *O. oeni* strain Alpha, a strain determined to be strongly inhibitory in the previous screening experiment. Using a 10 kDa dialysis membrane the two microorganisms were physically separated to determine whether physical contact was required for inhibition to occur. After inoculation *B. bruxellensis* populations in the control wine that did not undergo MLF initially decreased but entered exponential growth by day 18, reaching a population max of approximately 8×10^5 by day 56 (Figure 6). When *B. bruxellensis* and *O. oeni* cells were separated by a 10 kDa membrane, *B. bruxellensis* populations initially declined but soon entered exponential growth reaching a population maximum of approximately 1×10^6 by day 28 (Figure 6).When *B. bruxellensis* and *O. oeni* cells were not separated by a membrane, *B. bruxellensis* populations declined after inoculation to approximately 5×10^2 CFU/mL by day 4 and fell below detection threshold by day 28. *B. bruxellensis* populations did not rise above detection threshold until Day 56 reaching 1.6 $\times 10^6$ CFU/mL by day 70 (Figure 6). In summary, the physical separation of *O. oeni* from *B. bruxellensis* relieved the inhibition of *B. bruxellensis* by *O. oeni* that occurred when the two microorganism were in physical contact. These results indicate that inhibition is not due to nutrient depletion by *O. oeni* as nutrients could flow freely across the dialysis membrane. It is also highly unlikely that *B. bruxellensis* inhibition was due to the production of an inhibitory compound by *O. oeni* as unless the compounds were larger than 10kDa they would have passed through the dialysis membrane. Lactic acid bacteria are reported to produce inhibitory compounds such as the bacteriocin nisin (3-5 kDa) that is commonly used in food preservation schemes to protect from bacterial spoilage. However, the majority of lactic acid bacteria produced bacteriocins described in the literature are smaller than 10 kDa. In addition, if a bacteriocin larger than 10kDa was produced by *O. oeni*, it would have to be active against the eukaryote *B. bruxellensis*, something that has not been reported in literature for bacteriocins. Instead, the result from this study provide strong evidence that the inhibition of *B. bruxellensis* by *O. oeni* is due to cell-cell contact. This type of inhibition has been reported before for non-*Saccharomyces* yeast (Nissen et al. 2003) but this is the first report of this type of inhibition of *B. bruxellensis* by another microorganism.

To further investigate the nature of the inhibition resulting from MLF, *B. bruxellensis* strain UCD-2049 was inoculated into wine at different time points during MLF being conducted by *O. oeni* strain Alpha. *B. bruxellensis* populations in treatments that were co-inoculated with *O. oeni* behaved similarly to their controls, declining rapidly to below detection threshold by day 7, and not returning again by the end of the experiment on day 42 (Figure 7). *B. bruxellensis* populations in treatments that were inoculated after a partial MLF declined slightly faster than their control, with both control and treatment populations falling below detection threshold by day 11. The control recovered slightly by day 21, reaching a population of 2.3×10^2 CFU/mL, and continued to recover sluggishly before reaching a population of approximately 7×10^2 CFU/mL by day 39. *B. bruxellensis* populations in the partial MLF treatment wine did not recover until day 39, reaching a population of approximately 3×10^2 CFU/mL (Figure 7). *B. bruxellensis* populations in treatments that were inoculated after a 14 day MLF responded differently than their controls. *B. bruxellensis* in the control wine for this treatment had reached a population of approximately 9×10^4 CFU/mL by day 21, and had multiplied to approximately 4×10^6 CFU/mL

by day 35. *B. bruxellensis* populations in the MLF treated wine declined to below detection thresholds by day 21, and had not recovered by day 35 (Figure 7).

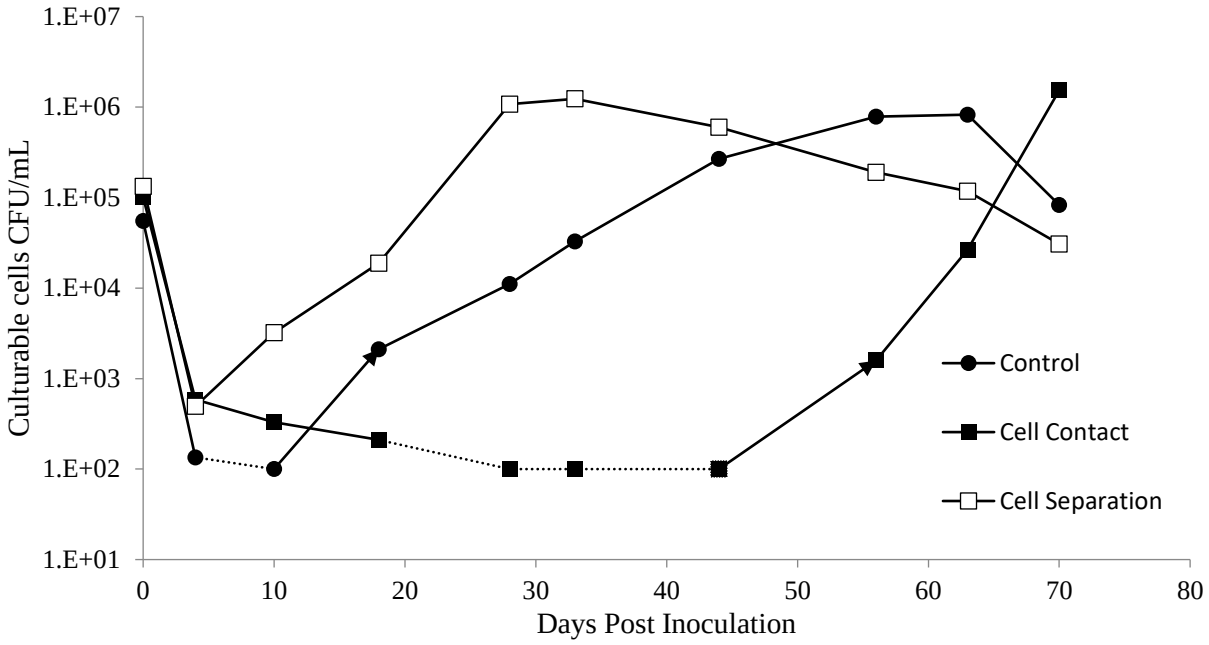

Figure 6. Culturable cells of *B. bruxellensis* UCD-2049 in Pinot noir wine that did not undergo MLF (control) and wine that underwent a MLF with *O. oeni* Alpha. *B. bruxellensis* and *O. oeni* cells were separated by a 10 kDa membrane (cell separation) or were not separated (cell contact).

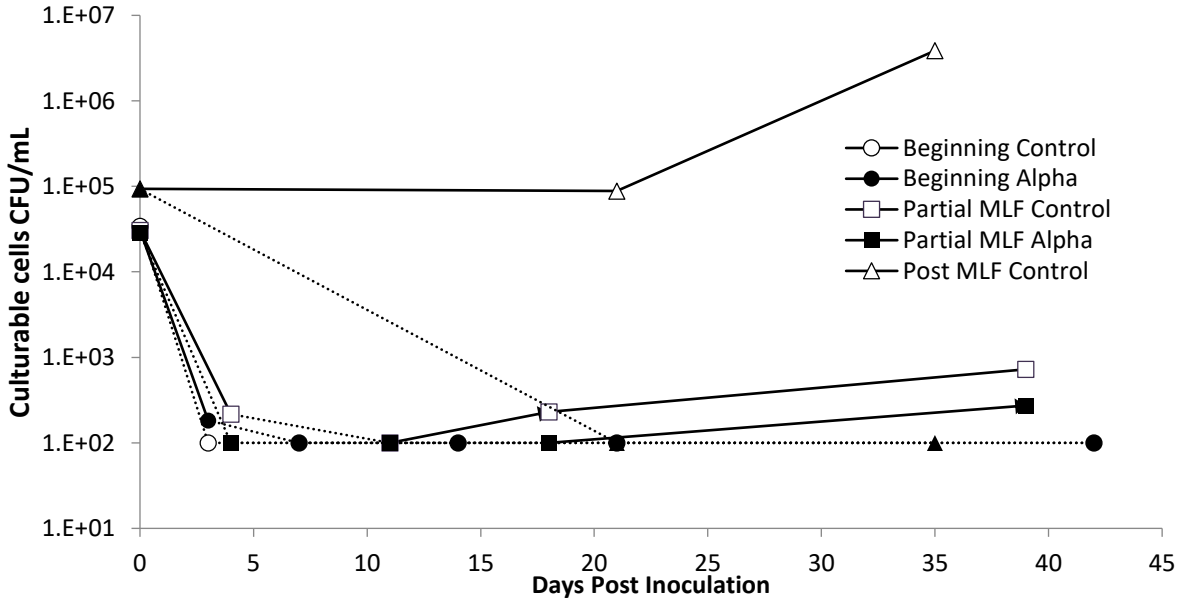

Figure 7. Culturable *B. bruxellensis* strain UCD-2049 cells in Pinot noir wine that did not undergo MLF (control) and wine that underwent MLF with *O. oeni* strain Alpha. *B. bruxellensis* was either co-inoculated (beginning), inoculated after partial MLF (partial), or inoculated at the end of MLF (post).

B. bruxellensis screening results:

The sensitivity of a range of *B. bruxellensis* strains to *O. oeni* was determined in 2018 Pinot noir. *O. oeni* strain Alpha was used as this strain was highly inhibitory to *B. bruxellensis* UCD-2049 in the previous *O. oeni* screening experiment. While *B. bruxellensis* UCD2049 culturability declined rapidly when inoculated into wine that had just completed MLF with *O. oeni* Alpha (Figure 8), growth of the other *B. bruxellensis* strains tested was not impacted (Figures 8&9). While there was some variation in growth between *B. bruxellensis* strains, similar growth occurred whether inoculated into wine that had or had not undergone MLF with *O. oeni* Alpha. At the completion of the screening experiment, wines were assessed for volatile phenols. As expected, the concentration of volatile phenols reflected growth of *B. bruxellensis* with minimal differences noted between strains grown in wine that had or had not undergone MLF (Table 4). The exception was *B. bruxellensis* UCD-2049 where much lower volatile phenols were present in wines that had undergone MLF with *O. oeni*. Again, this reflected the results from the growth study where *B. bruxellensis* UCD-2049 growth was inhibited by *O. oeni*.

Table 4. Concentration of volatile phenols (ug/L) in Pinot noir that had or had not undergone MLF with *O. oeni* Alpha and inoculated with various strains of *B. bruxellensis*

***Brettanomyces* strain**	4EG	4EP	4VG	4VP	Total volatile phenols
AWRI-1499	190.8	355.1	12.8	86.9	645.7
AWRI-1499 + *O. oeni*	191.7	365.9	11.1	98.2	666.9
Copper Mountain	221.2	391.3	14.6	90.9	718.1
Copper Mountain + *O. oeni*	250.3	425.9	19.2	122.5	817.9
i1a	246.9	463.9	17.1	94.1	822.1
i1a + *O. oeni*	212.1	462.4	14.9	106.7	796.1
UCD-738	220.7	415.2	14.9	95.8	746.6
UCD-738 + *O. oeni*	230.8	435.6	28.7	145.9	841.1
Y18	197.6	361.1	14.8	99.7	673.3
Y18 + *O. oeni*	210.5	374.7	15.7	113.6	714.6
UCD-2049	322.4	934.1	104.1	247.7	1608.3
UCD-2049 + *O. oeni*	8.9	<10	<10	<50	8.9

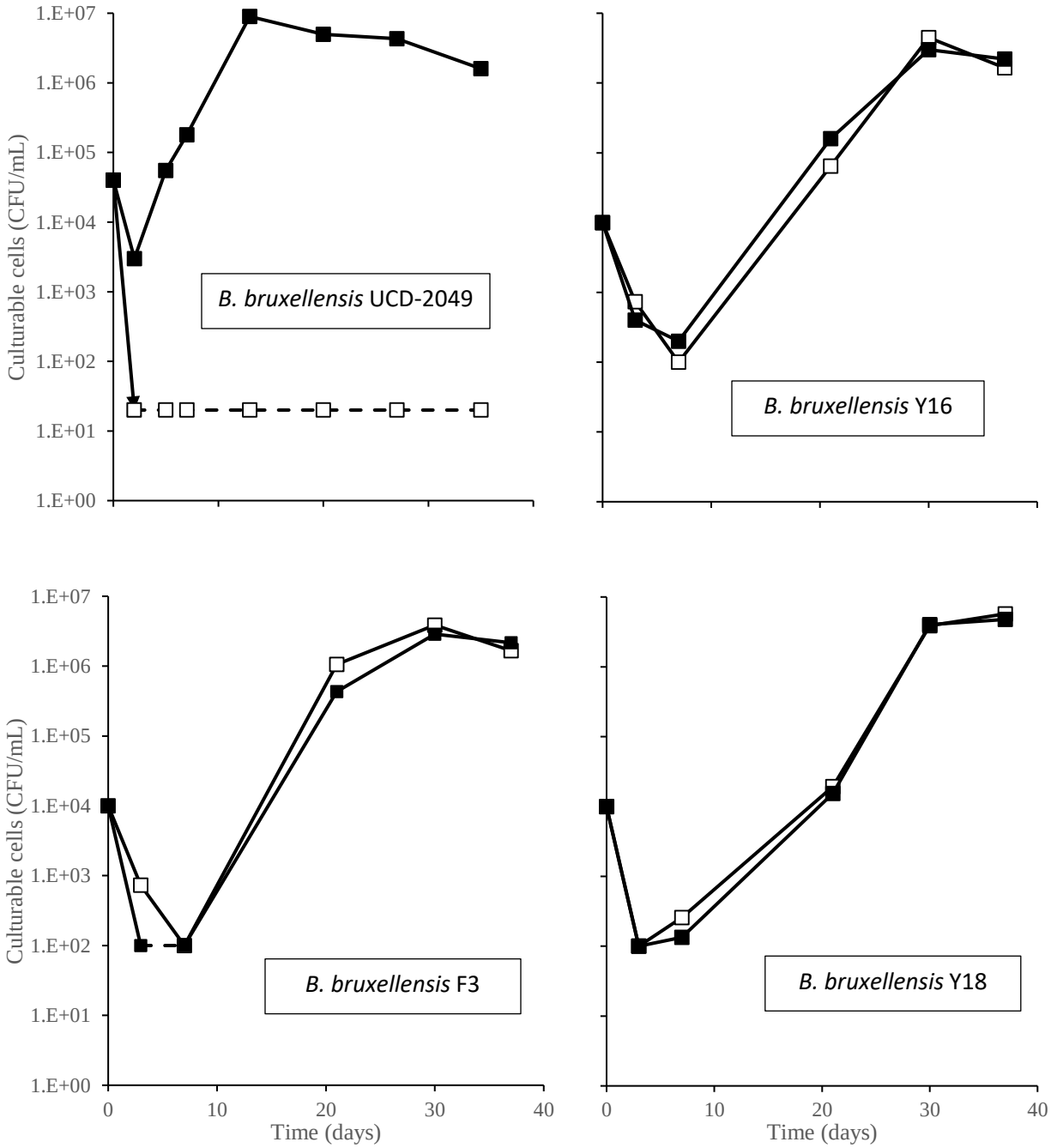

Figure 8. Growth of various *B. bruxellensis* strains in Pinot noir wine that has (□) or has not (■) undergone MLF with *O. oeni* Alpha.

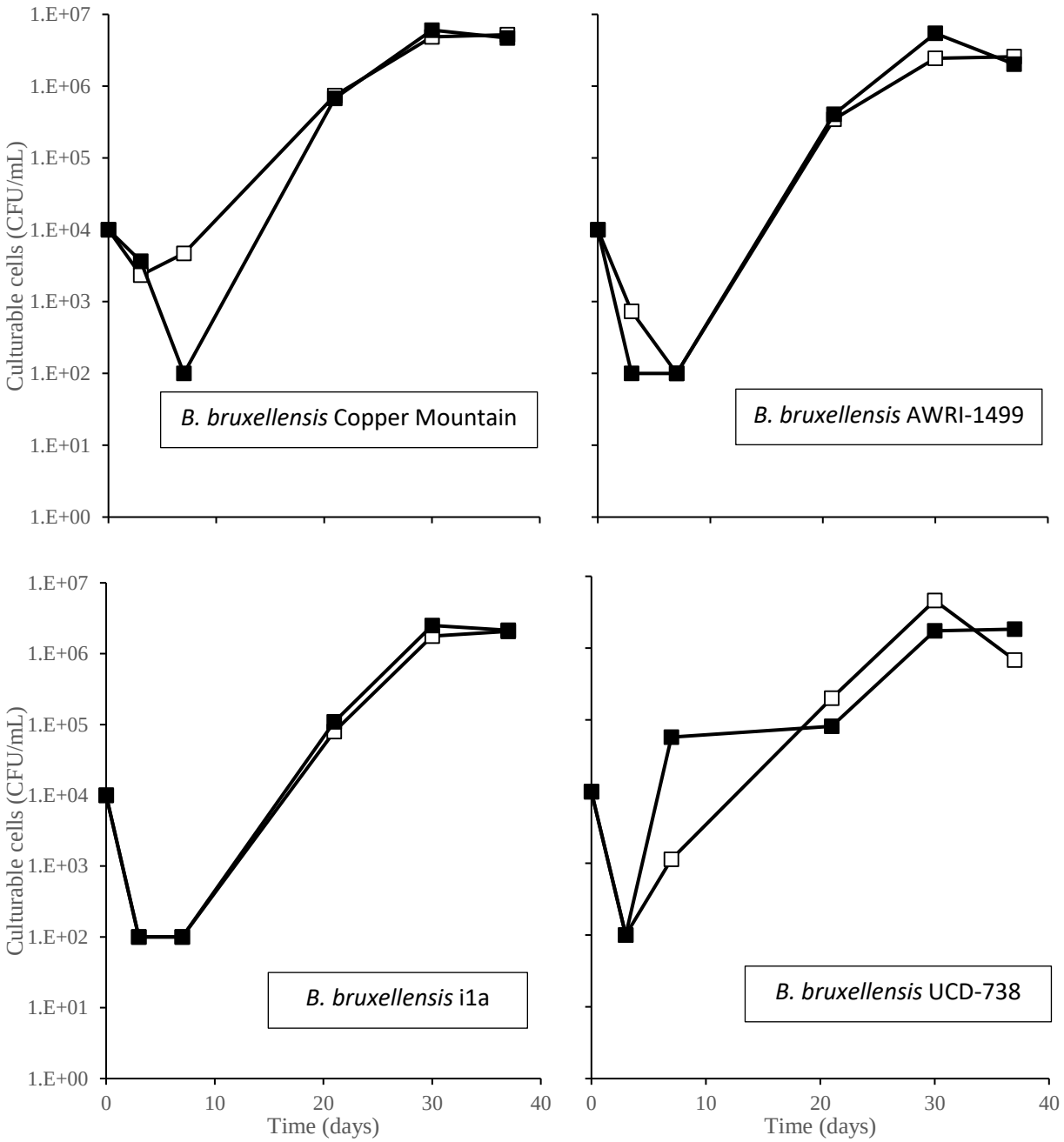

Figure 9. Growth of various *B. bruxellensis* strains in Pinot noir wine that has (□) or has not (■) undergone MLF with *O. oeni* Alpha.

Effect of ethanol impact on *B. bruxellensis* inhibition by *O. oeni*

Why *B. bruxellensis* strain UCD2049 is sensitive to *O. oeni* while the other *B. bruxellensis* strains were not is unknown at this point. One possibility is that the inhibition of *B. bruxellensis* by *O. oeni* is influenced by wine conditions such as pH and ethanol. During the screening studies, wine pH ethanol content was adjusted to make it conducive to the growth of *B. bruxellensis* (pH 3.60 & 13% v/v ethanol). Additional experiments were therefore conducted in wines adjusted to higher or lower ethanol content to determine if there is a synergistic effect of ethanol and MLF. The effect of ethanol was investigated initially as *B. bruxellensis* strains are known to have different tolerances to ethanol (Strum et al. 2014, Oswald and Edwards 2017, Edwards and Oswald 2018). *B. bruxellensis* strains UCD-2049, Copper Mountain, and AWRI-1499 were inoculated into wines adjusted to high or low EtOH concentrations (12.5 and 14.0% EtOH v/v respectively) that had or had not undergone MLF with *O. oeni* strain Alpha. Wines were pH adjusted after MLF to minimize the impact this variable could have on *B. bruxellensis* growth. In the low EtOH wines, *B. bruxellensis* strain UCD-2049 grew well in low EtOH wine whether it was inoculated into wine that had undergone MLF or not (Figure 10A). This is in contrast to what was observed earlier (Figures 3-5) for UCD-2049. However, the earlier results were seen in Pinot noir wine that had been adjusted to 13% EtOH. In the higher ethanol wine (14%), *B. bruxellensis* UCD-2049 populations declined quickly in both the MLF treated wine and in the control, with populations falling to $1x10^2$ CFU/mL in the control and below detection threshold in the MLF treatment. *B. bruxellensis* populations in the control and MLF treated wines with populations in the MLF treated wines not recovering at any time during the 67 days of the experiment (Figure 10A). *B. bruxellensis* populations in the control wine that had not undergone MLF began to recover near the end of the experiment with $2x10^3$ CFU/mL being detected on day 67 (Figure 10A).

B. bruxellensis Copper Mountain (CM) grew well in the low EtOH control wines and reached a population maximum of approximately 6×10^6 CFU/mL by day 16 (Figure 10B). In contrast, populations in the low EtOH wine that had undergone MLF decreased after inoculation to approximately $1x10^3$ CFU/mL by day 4. However, the *B. bruxellensis* populations recovered and reached a similar population to the control by day 16 (Figure 10B). In the high EtOH wine, *B. bruxellensis* CM populations in both the control and MLF wine declined soon after inoculation but recovered in the MLF wine reaching a maximum of approximately $3x10^6$ CFU/mL by day 49 (Figure 10B). In the control wine the *B. bruxellensis* CM populations remained low until recovering by the end of the experiment.

B. bruxellensis AWRI-1499 populations in low EtOH wine for both the control and MLF treatments behaved similarly. For both treatments, *B. bruxellensis* AWRI-1499 entered exponential growth by day 9, reaching a population maximum by day 48 (Figure 10C). While no differences between *B. bruxellensis* AWRI-1499 populations were observed between the control or MLF treatments was at low EtOH, a difference was noted in the high ethanol wine (Figure 10C). *B. bruxellensis* AWRI-1499 populations in the control wines declined to approximately 4×10^3 CFU/mL by day 4 but had entered exponential growth by day 9, reaching a population maximum of 5×10^6 CFU/mL by day 49. In high EtOH wine that had undergone MLF with *O. oeni* Alpha, *B. bruxellensis* populations declined to approximately 4×10^2 CFU/mL by day 9, and recovered slower than the populations in the control. For example, 16 days after inoculation *B. bruxellensis* populations in the control had reached approx. 2×10^5 CFU/mL while in wine

that had undergone MLF the population was only approx. 1 x 10^3 CFU/mL (Figure 10C). *B. bruxellensis* populations in wine that underwent MLF did not reach similar populations to the control until day 49.

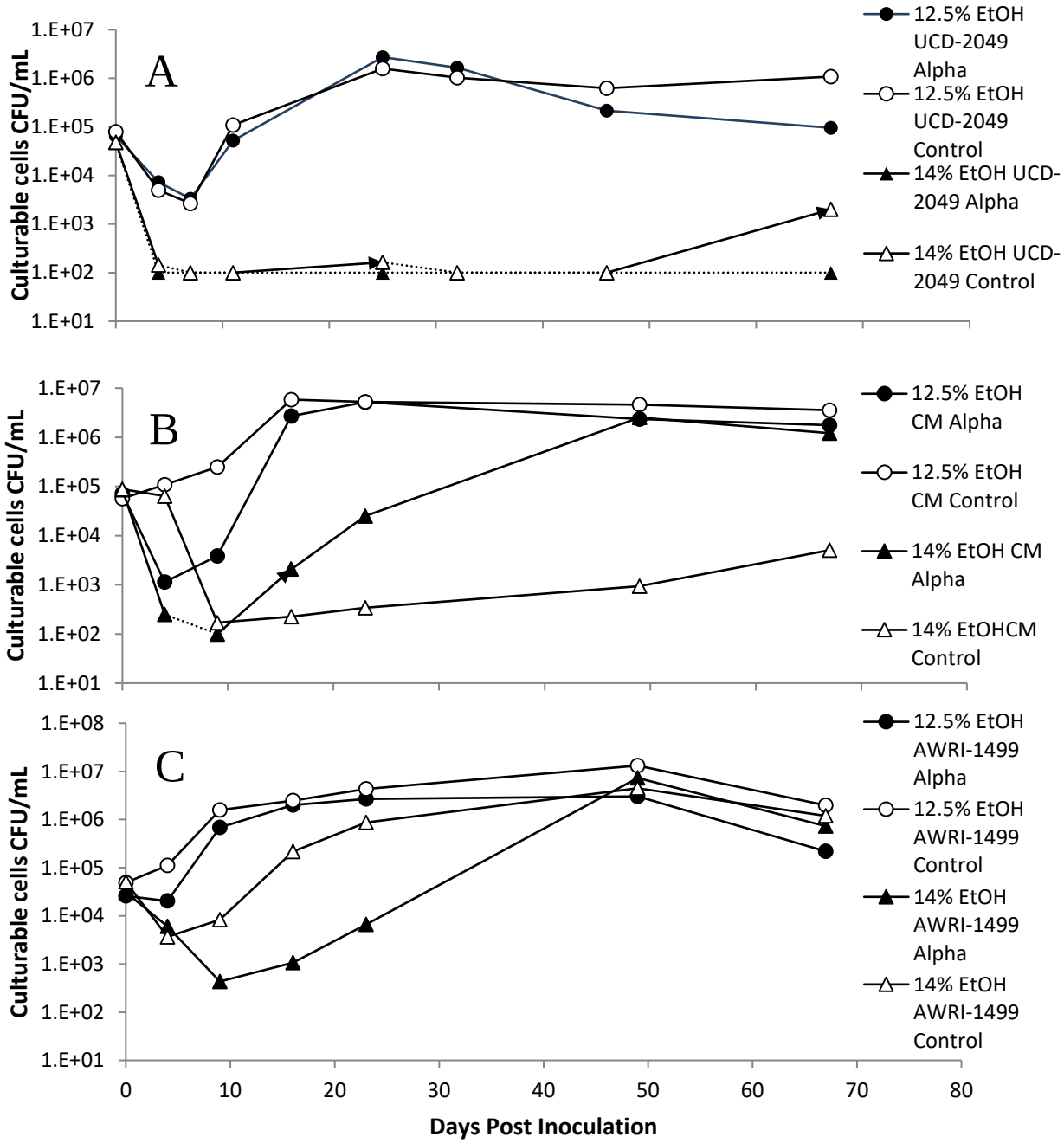

Figure. 10 Growth of *B. bruxellensis* strain UCD-2049 (A), Copper Mountain (B), or AWRI-1499 (C) in Pinot noir wine adjusted to 12.5% EtOH v/v or to 14% EtOH v/v that has (Alpha) or has not (Control) undergone MLF with *O. oeni* strain Alpha.

VI. OUTSIDE PRESENTATIONS OF RESEARCH

A presentation of results from this research was planned for the 2020 American Society for Enology and Viticulture annual meeting (Portland, OR). Due to the pandemic this meeting was cancelled. Research results instead will be presented at the 2021 American Society for Enology and Viticulture annual meeting in Monterey CA. An update on this project will be presented at the 2021 Oregon Wine Symposium.

VII. RESEARCH SUCCESS STATEMENTS:

To date, this research has resulted in a number of significant findings regarding interactions between *Oenococcus* and *Brettanomyces bruxellensis*. Firstly, *B. bruxellensis* UCD-2049 growth was strongly inhibited when inoculated into wine at the end of MLF. All *O. oeni* strains used to conduct MLF had a similar initial effect on *B. bruxellensis* growth but *O. oeni* strain variability was observed during the course of the experiment. For example, some *O. oeni* strains so strongly inhibited *B. bruxellensis* that populations were not detected throughout the experiment while *B. bruxellensis* growth was observed near the end of the experiment for other strains. These strain differences suggest that some *O. oeni* strains may be more effective than others at suppressing *B. bruxellensis* growth than others, and given the practical implications, merits additional study. How *O. oeni* inhibits *B. bruxellensis* was also determined and is likely due to cell-cell contact between the two microorganisms rather than nutrient depletion or production of inhibitory compound(s). A further significant finding was that not all *B. bruxellensis* strains were as susceptible to inhibition by *O. oeni* as *B. bruxellensis* strain UCD-2049 was. A possible explanation for this strain variance was explored by testing whether ethanol tolerance impacted *B. bruxellensis* inhibition by *O. oeni*. Results demonstrated that ethanol tolerance differences between *B. bruxellensis* strains could impact their inhibition by *O. oeni*. For example, strain UCD2049 was not inhibited by *O. oeni* in wine at 12.5% ethanol but was inhibited in 13% and 14% ethanol wine. In contrast, a more ethanol tolerant *B. bruxellensis* strain, AWRI-1499, was not inhibited by *O. oeni* in 12.5% or 13% (v/v) Pinot noir wine but some growth repression was observed at in 14% (v/v) ethanol wine. The results of this study show that MLF conducted by *O. oeni* may offer some protection from spoilage by *B. bruxellensis,* though any protection conferred by MLF is likely to vary depending on *B. bruxellensis* strain and wine chemistry. Overall, the effects of MLF on *B. bruxellensis* are not such that they could be considered a standalone spoilage prevention strategy by winemakers. Instead, MLF could be considered as an additional hurdle that could be used to lower the risk of wine spoilage by *B. bruxellensis,* particularly during the period of time between the end of MLF and the addition of SO2 by the winemaker.

VIII. FUND STATUS:

Funds have been spent to support a graduate student and an undergraduate student who are conducting the experiments. Funds have been spent for winemaking, HPLC supplies for hydroxycinnamic acid analysis, microbiological media, and volatile phenol analysis by GC-MS. The majority of remaining funds are allocated for salary and volatile phenol analysis.

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