Innovative Use of Non-*Saccharomyces* in Bio-Protection: *T. delbrueckii* and *M. pulcherrima* Applied to a Machine Harvester

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Summary

Goals: This study addresses the increasing demand for "natural" and certified organic wines, along with the need for improved worker safety. Winemakers continue to search for alternatives to SO_2 as an antioxidant and antimicrobial agent. This study compares the use of blended non-*Saccharomyces cerevisiae* yeasts—*Torulaspora delbrueckii* (*Td*) and *Metschnikowia pulcherrima* (*Mp*)—as antimicrobial agents to a standard addition of SO_2 on Cabernet Sauvignon. This fruit possesses over 10 times the normal microbial flora typically found in California. In conjunction with this comparison study, a proof of concept prototype illustrates the use of a novel spray method for the application of these non-*Saccharomyces* yeasts onto a grape machine harvester for bioprotection.

Key Findings:

Research Winery:

- Overall, the blended yeasts performed better than a standard addition of SO_2 at controlling wine spoilage organisms on compromised fruit.
- Organisms related to wine spoilage responded differently to *Td/Mp* than to SO_2. The *Td/Mp* treatment exhibited an advantage over the SO_2 treatment. The *Td/Mp* treatment appeared to work best against *Zygosaccharomyces*, *Lactobacillus kunkeei*, *Hanseniaspora uvarum*, and acetic acid bacteria. It was less effective against *Pediococcus* and other *Lactobacillus* species.
- Different stages of the trial fermentations were affected differently by *Td/Mp* and SO_2. The *Td/Mp* populations performed best during prefermentation and the early stages of fermentation.
- *Td/Mp* showed an antagonistic effect on microorganisms responsible for wine spoilage. There were fewer microorganisms related to spoilage growing in the three bioreactors with non-*Saccharomyces* species than in the bioreactors acting as experimental controls with 60 mg/L SO_2 added during processing.
- *Td/Mp* treatment increased the implantation capacity of *S. cerevisiae* compared to the use of SO_2. Using identical inoculation rates of *S. cerevisiae*, we found more *S. cerevisiae* cells growing in the *Td/Mp* bioreactors than in the bioreactors treated with SO_2. Furthermore, we observed greater population reduction and fewer cells/mL of *S. cerevisiae* at the end of fermentation.

Field Trial:

- A reduction in spoilage microorganisms occurred when using *Td/Mp* directly applied to the harvester.
- Applying *Td/Mp* yeasts to the grape harvester reduced aromas related to volatile acidity coming from the machine.

Impact and Significance: The use of *Td/Mp* yeasts provides an alternative to SO_2 for controlling the growth of organisms related to wine spoilage. Incorporating these yeasts as a bioprotectant by applying them during the harvest and transport processes reduces the risk of detrimental microbial organisms in the harvested fruit, juice, and wine.

Key words: *Metschnikowia pulcherrima*, microbial populations, *Torulaspora delbrueckii*, vineyard management, yeast ecology

Overview

Winemakers continue to search for alternatives to SO_2 in winemaking. Many producers seek to reduce SO_2 to take advantage of market opportunities in the "natural" wine movement by following organic certification guidelines, to improve worker safety, and to utilize natural products known to have similar efficacy to synthetic products for targeted applications. Winemakers currently use SO_2 to control microbial growth. However, it is also a powerful antioxidant and inhibits browning reactions. Because the properties of SO_2 allow many different winemaking applications,[1] reducing or completely removing SO_2 from winemaking requires an investigation of each processing step, beginning with grape harvesting and transport.

Bioprotection is a relatively new term and emerging concept in several food industries.[2,3,4] In this study, the term refers to a natural agent that controls the growth of unwanted organisms through ecological processes such as competition. This study examines the efficacy of a blend of *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* (*Td/Mp*, 1:1 ratio by cell count) as a bioprotection agent. This mixed culture is a commercial product currently used by winemakers. The nature and impact of these yeast species on winemaking is a current area of research at universities and developmental laboratories worldwide.[2,5,6]

This study investigates *T. delbrueckii* as a co-inoculum for the bioprotection of grapes and juices. Some data suggest that the effectiveness of *T. delbrueckii* as a bioprotectant depends on the matrix.[21] Another study illustrates that *T. delbrueckii* populations show much less severe losses in viability during the early stages of fermentation than other non-*Saccharomyces* species, making it an ideal choice as a competitive species for inoculation during prefermentation.[7,8]

In addition to its use for bioprotection, *T. delbrueckii* is known to enhance the aromatics of wine produced in a cofermentation with *Saccharomyces cerevisiae* and is widely used in industry for this purpose.[9] Evidence from the University of Bordeaux found that co-inoculation with *T. delbrueckii* and *S. cerevisiae* produced 54% less volatile acidity and 60% less acetaldehyde than inoculation with *S. cerevisiae* alone.[10] Other studies show a positive sensory impact of cofermentation in both sequential and simultaneous mixed cultures of *T. delbrueckii* and *S. cerevisiae*, most notably an increase in fruity aroma linked to specific esters and tropical aroma profiles releasing volatile thiols (3SH and 4MSP).[9,11]

T. delbrueckii is a well-documented yeast and contributes to mouthfeel sensations along with many other non-*Saccharomyces* species.[10,11,12,13,14] Many of the mechanisms for increased mouthfeel result from an increase in mannoprotein content from the cell.[13] *T. delbrueckii* is also known to metabolize sugar to produce alternative compounds such as glycerol or pyruvic acid via the Crabtree effect.[15] An additional study reveals an impact regarding the modulation of astringency resulting from *T. delbrueckii* fermentation.[16]

The use of *M. pulcherrima* as a biological control agent is possible thanks to its ability to produce the natural antimicrobial compound pulcherrimin. This compound is an insoluble red pigment with antifungal activity.[6] Pulcherrimin has been shown to deplete iron in growth media, which in a fermentation could result in inhibition of organisms requiring iron for growth.[17] This mechanism of iron depletion occurs via the precipitation of iron(III) ions caused by an interaction with pulcherriminic acid, a precursor of pulcherrimin secreted by *M. pulcherrima.*[6] Several microorganisms exhibit inhibitory effects from pulcherrimin, including *Candida tropicalis*, *Candida albicans*, *Brettanomyces/Dekkera*, *Hanseniaspora*, *Pichia*, and *Botrytis cinerea*. *S. cerevisiae* appears unaffected by this antimicrobial activity.[3] In addition, some strains of *M. pulcherrima* produce a killer factor to suppress growth of killer-sensitive organisms. *M. pulcherrima* is also described as a biofungicide capable of reducing *B. cinerea* on postharvest fruits via nutrient competition.[18]

Uninoculated non-*S. cerevisiae* yeasts, often called "wild" yeasts, are commonly associated with the production of ethyl acetate and negative sensory characteristics.[14] However, mixed cultures of *T. delbrueckii* and other yeasts are known to produce positive aromas without the negatively associated sensory attributes.[14] *M. pulcherrima* is known to produce high concentrations of esters[19,20,21,22,23] and also possesses β-glucosidase activity, which catalyzes the release of varietal aromas.[5] An additional study illustrates that co-inoculations of *M. pulcherrima* with *S. cerevisiae* reduce the total amount of acetic acid in the final wine.[4] Wines inoculated initially with *S. cerevisiae* and *M. pulcherrima* show contributions of 2-phenylethanol and several esters in the final product.[12]

In this study, we validated the bioprotective nature of the mixed cultures at the University of California Davis Research Winery. The commercially available bioprotection product is compared to a standard addition of SO_2 on fruit possessing a high number of microbial organisms related to wine spoilage.

In addition, we built a prototype blaster as a novel method for applying the dry mixed culture onto a mechanical grape harvester in the vineyard. The aim was to

encourage the growth of specific inoculated yeast rather than oxidative spoilage organisms, after a winemaker complained that the harvester "always smells like VA (volatile acidity, typically acetic acid and ethyl acetate) during harvest." We quantified the impact of bioprotection in both trials by evaluating the microbiota of fermentation using qPCR Scorpions from ETS Laboratories in Saint Helena, CA and performed a statistical analysis of the resulting ecological population data sets.

Major Observations and Interpretations

The *Td/Mp* inoculation was better than the addition of SO_2 at controlling organisms related to wine spoilage from highly compromised fruit. The acetic acid bacteria level found in the fruit was 10 times the average value measured by ETS Laboratories during the 2018 harvest based on microbiota evaluation using qPCR from ETS. Physical examination of the fruit revealed excessive damage resulting in macerated grapes and grape skin wounds consistent with bird damage. Experienced winemakers noted aromas related to a compromised crop and significant insect populations on the fruit.

Sampling occurred on days 1, 4, 6, 8, and 15. We decided before the experiment that we would seek greater resolution of data points during the prefermentation and early fermentation stages. Thus, the majority of sampling occurred during the early stages of fermentation. Furthermore, we had to sample on days when the research winery was open and took extra care not to interfere with student and faculty experimentation.

Our exploratory approach to the data included examining the raw data, searching for outliers, transformations of the data, descriptive statistics, and robust statistical analysis. We seek to provide a narrative for the data while presenting our findings in simple terms. To prove the statistical significance of our work, we also provide some information on our statistical analysis and results.

For the sake of simplicity, we include the data as a raw representation of the sum of cells detected (Figure 1). Note that the cell counts are the sum of all the measurements taken for each organism. We also include the organismal response to the treatments in this figure. In this way, the raw data is presented for each spoilage organism, as well as for the overall number of spoilage organisms. Notice some organisms contribute more to the total amount of organisms than others. This provides the first glimpse of what is known as an outlier.

If we divide the data into subsets, we can see how the phases of fermentation influence the growth of the organisms. The protective *Td/Mp* populations only survive
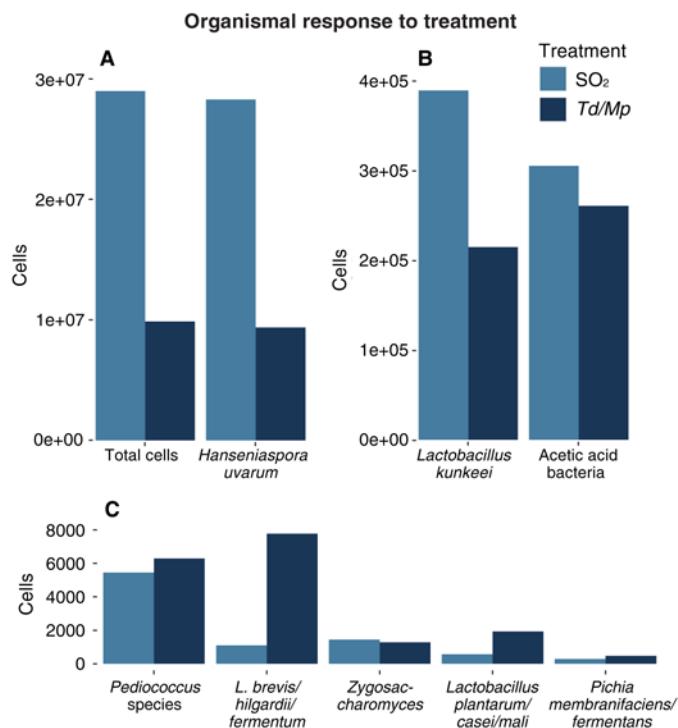

Figure 1 Overall, the addition of *Td/Mp* (a blend of *Torulaspora delbrueckii* and *Metschnikowia pulcherrima*) performed better than a standard addition of SO_2 at controlling wine spoilage organisms in the research winery. Note that the cell counts are the sum of all the measurements taken.
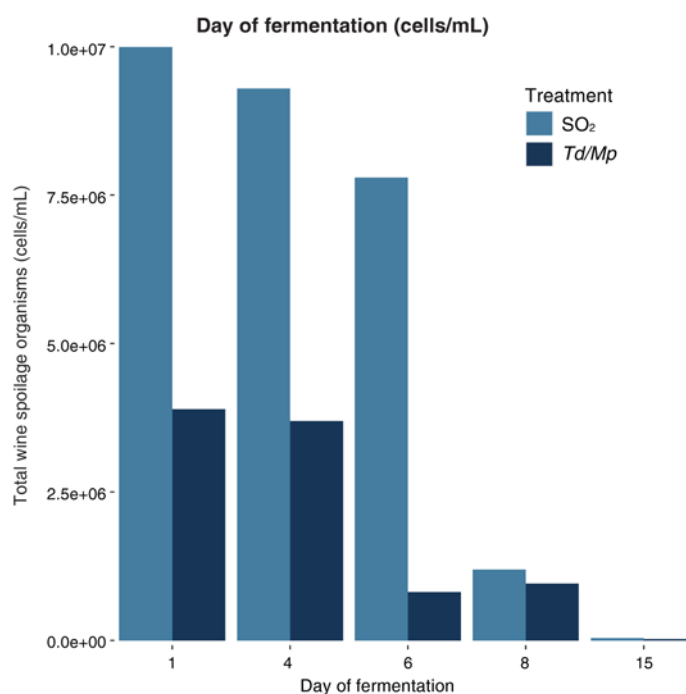

Figure 2 Different stages of the laboratory fermentation are affected differently using *Td/Mp* (a blend of *Torulaspora delbrueckii* and *Metschnikowia pulcherrima*) versus SO_2. This graph depicts the sum of all the microorganisms listed by day.

the initial stage of fermentation (Figure 2). Once the alcohol of the system reaches between 3 and 7%, they die. The same is true for some of the spoilage organisms, which possess various levels of ethanol tolerance. In the case of the controlled bioreactors, after day 8, many of the microorganisms related to wine spoilage had died. Using the *Td/Mp* populations as a protective factor against unwanted organisms worked better than SO_2 during the earliest stages of fermentation.

In Figure 1, we split the data into subgroups labelled A, B, and C. These subgroups represent different levels of contribution to the overall total number of spoilage organisms. One population contributed more than any of the other populations. In statistical terms, we call this population an outlier in subgroup A. Interestingly, the addition of SO_2 resulted in a marked increase in populations of *Hanseniaspora uvarum* compared to *Td/Mp* treatment. *H. uvarum* dominated the control fermentation, reaching ~10 million cells/mL during the initial stages of fermentation. In comparison, *Td/Mp* treatment resulted in much less *H. uvarum*, with cell counts reaching 4 million cells/mL. *H. uvarum* cells appeared to be uninhibited by the addition of SO_2. A record of each organismal population response is available in Table 1. Finding outliers is often done with a Monte Carlo simulation. As we explored the data, we took note of the outlier and instead of focusing on it—contemplating its removal or reason of existence—we simply included it as part of the fermentative system of organisms and moved to a transformative approach to data mining.

Transformations are a key part of data analysis, in much the same way as outlier detection algorithms. Transforming the data allows us to change the dimensionality of the data and examine the data set in more detail. A common technique for understanding the sanitary effects of agents in microbiology is known as log-kill. The FDA uses guidelines based on log-kill or log-change to determine the efficacy of sanitation and sterilization procedures. Transforming the data into log-change allows us to understand the relative rate of growth and death of an organism.

It is essential for the protective effect of the *Td/Mp* not to interfere with the ability of *S. cerevisiae* to complete the alcoholic fermentation. We examined the *S. cerevisiae* populations during the different stages of fermentation and calculated the log-change throughout the fermentation. In doing so, we examined the function of *S. cerevisiae* in terms of biomass development, reproductive rates, and cell death rates. Using log-change, we were able to describe changes in the dynamic system that provide better insight into rate changes than do cell counts alone. We found that *Td/Mp* treatment resulted in better implantation for *S. cerevisiae* compared to SO_2 treatment (Figure 3). Better implantation indicates more substantial rates of growth and more biomass.

We also noted more pronounced cell death in the late stages of fermentation. We suspect that the redox potential of the system may also be affected by the presence of *Td/Mp* populations and intend to study this further in

Table 1 Total cells for each tested organism. *Td/Mp*, a blend of *Torulaspora delbrueckii* and *Metschnikowia pulcherrima*.

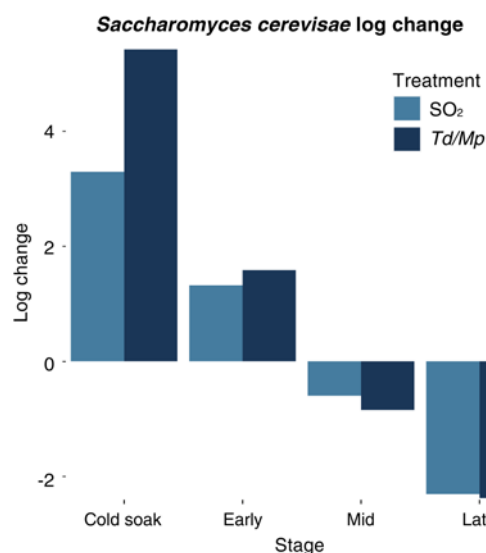| Treatment	Organism	Total cells counted during fermentation
SO_2	Acetic Acid Bacteria	305,040
Td/Mp	Acetic Acid Bacteria	260,620
SO_2	*Hanseniaspora uvarum*	28,309,780
Td/Mp	*H. uvarum*	9,387,320
SO_2	*Lactobacillus brevis/hilgardii/fermentum*	1101
Td/Mp	*L. brevis/hilgardii/fermentum*	7763
SO_2	*Lactobacillus kunkeei*	388,900
Td/Mp	*L. kunkeei*	214,650
SO_2	*Lactobacillus plantarum/casei/mali*	571
Td/Mp	*L. plantarum/casei/mali*	1931
SO_2	*Pediococcus*	5450
Td/Mp	*Pediococcus*	6290
SO_2	*Pichia membranfaciens*	292
Td/Mp	*P. membranfaciens*	483
SO_2	*Zygosaccharomyces*	1451
Td/Mp	*Zygosaccharomyces*	1291

Figure 3 The *Saccharomyces cerevisiae* with *Td/Mp* (blend of *Torulaspora delbrueckii* and *Metschnikowia pulcherrima*) showed better implantation compared to an identical inoculation with SO_2. This graph illustrates the growth (positive values) and death (negative vales) of *S. cerevisiae* as the logarithmic change between the stages of fermentation.

future collaborative endeavors with our research partners. Furthermore, the rapid die-off of the *S. cerevisiae* may inhibit its ability to contribute off-aromas and flavors in the middle and late stages of fermentation.

After exploring the microbiota data with descriptive statistics, searching for outliers, determining the data distribution, and performing various transformations, we sought to determine whether the resulting data from our study is conclusive. To do this, we needed to define statistical significance. We set out to answer the question: "Can we illustrate a significant advantage of using a

Figure 4 Yeast Blaster Prototype One applicator for dry addition of yeast for bioprotection. The unit includes a canister for holding dry product and two gas attachments in high-pressure and low-pressure serviceable systems. Backpack unit not shown.

group of non-*Saccharomyces* yeasts compared to a standard addition of SO_2?"

We had already performed an exploratory dive into the data by examining our assumptions from analysis of variance (ANOVA) Type II, including random sampling, normal distribution, and balanced experimental design. This data is not shown but is provided through a link to Github in the final section of this report. We made a log transformation (*S. cerevisiae*, mentioned above) and examined outliers of the data. We also used several data mining methods commonly found in geometric data analysis or data science. However, due to the scope of this report, we do not discuss the details of the data mining approach in more depth. Instead, we simply provide raw data numbers and validate our results using statistics.

Once significance was determined with ANOVA Type II in the research winery, we performed an additional proof of concept study at a winery where the winemaker had complained: "My harvester smells like VA!" Knowing that the machine harvester might be serving as a vector for spoilage organisms in the vineyard, we constructed an air-powered applicator named Yeast Blaster Prototype One (Figure 4). Yeast Blasters, blaster components, and build-your-own blaster plans are available from Laffort USA in Petaluma, CA. The field trial consisted of daily applications of *Td/Mp* on the harvester at the end of a washdown procedure (Table 2). This trial illustrated a proof of concept, but we need to perform significance testing on the application, including multiple replicates with several different harvesters and iterations of a field trial.

Table 2 Bacteria and yeast populations on machine harvester as measured in the first fruit harvested at the commencement of shift every week for three weeks, with the first analysis (Week 1) done before *Td/Mp* (a blend of *Torulaspora delbrueckii* and *Metschnikowia pulcherrima*) application.

Harvester application trial (cells/mL)	Pre-treatment	Treatment	
	Week 1	Week 2	Week 3
Bacteria			
Acetic acid bacteria	22,000	7800	4600
Oenococcus oeni	520	700	240
Lactobacillus brevis, L. hilgardii, L. fermentum	40	50	300
Lactobacillus plantarum, L. casei, L. mali	4800	1300	720
Lactobacillus kunkeei	40	<40	230
Pediococcus species	290	120	230
Yeasts			
Hanseniaspora uvarum	26,800	7100	10,900
Zygosaccharomyces species	40	50	50
Pichia membranifaciens, P. fermentans	230	100	0
Brettanomyces bruxellensis	<40	<40	<40
Yeasts and bacteria			
Sum of bacteria	27,690	9970	6320
Sum of yeasts	27,070	7250	10,950
Sum of yeasts and bacteria	54,760	17,220	17,270

According to the winemaker, application of *Td/Mp* to the grape harvester reduced the smell of VA coming from the machine. We also noted decreases in cells per mL in fruit coming from the harvester (Table 2). However, field trials have many more variables than do validations in a research winery. The second and third weeks of the trial had average low temperatures nearly 2°F colder than those during the first week, which may have affected the results. This preliminary study provides anecdotal evidence as a reason for a continuation of our research with multiple harvesters.

Broader Impact

This study validated product claims that using *Td/Mp* as a bioprotectant can reduce the need for SO_2 in winemaking. The microbiota investigation used qPCR data to quantify a greater reduction of spoilage-related organisms in wine from grapes treated with *Td/Mp* versus grapes treated with SO_2. Specific organismal responses to treatment are found in Figure 1. The study also tested a novel dry application of *Td/Mp* with the Yeast Prototype Blaster One (Figure 4). Early stages of product validation in vineyard trials showed promise in North America. The use of *Td/Mp* to reduce the amount of SO_2 needed during winemaking is thus a validated process that will continue to gain traction as more wineries and vineyards seek out new agricultural processes related to bioprotection.

This study examined the role of non-*Saccharomyces* yeasts as a bioprotectant but only tested the responses of 10 organisms. Published peer-reviewed data indicate that the process may also work for *B. cinera*.[17] In addition, several winemakers have inquired about the use of *Td/Mp* against powdery mildew. We are currently considering developments into vineyard applications (Figure 5).

Figure 5 Proposed blaster use in vineyard for Botrytis control.

A continuation of this study would link these topics in a future experiment. The use of *Td/Mp* as a bioprotectant in the vineyard during harvest provides the earliest protection against microbial spoilage.

Experimental Design

Prefermentation. In this portion of the trial, we tested the efficacy of a mixed inoculation of *Td* and *Mp* packed in a 1:1 ratio by cell count. The trial used Cabernet Sauvignon fruit possessing a high microbial load in an experiment involving a control and two replicates. We used ~1 ton of Cabernet Sauvignon, donated from Elk Grove AVA and processed at the University of California, Davis. Harvest occurred on the night of 22 Sept 2019. The grapes received a 30 mg/L addition of SO_2 during harvest and were then incubated in a chilled room for three days before fruit processing.

The harvested Cabernet Sauvignon fruit was then incrementally transferred to six bioreactors. Each fermenter possessed 132.5 L (35 gallons) of crushed Cabernet Sauvignon after filling. The group of six bioreactors included two sets, one for control and one for the mixed bioprotectant culture. The control tanks received a 30 mg/L SO_2 addition commonly found in a practical winery setting with compromised fruit. Thus, the control tanks received a total of 60 mg/L SO_2. The mixed-culture tanks received no additional sulfur. Instead, they received 50 g/hL of *T. delbrueckii* and *M. pulcherrima* in a 1:1 ratio by cell count. All tanks were set to 56°F for a three-day cold soak. The experimental cellar team adjusted the yeast assimilable nitrogen to 250mg/L and the titratable acidity to 6.0 g/L. We ran a complete juice panel and SCORPIONS for Yeast and Bacteria at ETS Laboratories. Sampling included equal amounts from each of the bioreactors. The initial juice chemistry is given in Table 3.

Primary fermentation. The temperature of the jackets was raised to ~65°F at the end of 27 Sept 2019, after the three-day cold soak. The control bioreactors were inoculated on 28 Sept 2019 with a strain of *S. cerevisiae*

Table 3 Initial juice chemistry from bioreactor fermentations at UC Davis Research Winery. *Td/Mp*, a blend of *Torulaspora delbrueckii* and *Metschnikowia pulcherrima*.

Analysis	SO$_2$	*Td/Mp*
Titratable acidity	3.4 g/L	3.5 g/L
pH	3.67	3.63
Volatile acidity (acetic)	0.07 g/L	0.09 g/L
L-malic	0.71 g/L	0.73 g/L
Brix	23.9 degrees	23.7 degrees
Glucose + fructose	249 g/L	242 g/L
Yeast assimilable nitrogen	129 mg/L (as N)	127 mg/L (as N)

(Laffort Zymaflore FX10). The *Td/Mp* trial fermenters were inoculated with *S. cerevisiae* on 30 Sept 2019 after a near 3°F drop in Brix. Both inoculations included 20g/hL of rehydration nutrient (Laffort Superstart Rouge). The tank jacket temperature setting corresponded to 75°F at inoculation.

Postfermentation. After alcoholic fermentation was complete, the individual bioreactors had their contents drained and combined into two stainless steel drums separated as trial and control. An addition of *Oenococcus oeni* ensured that malolactic fermentation took place (Laffort Lactoenos SB3 Direct). The research winery laboratory concluded postfermentation with a verification of complete malic to lactic acid conversion and the addition of 50 mg/L SO_2. The final wine chemistry is listed in Table 4.

Preliminary prototype application. Following the development of dosing protocols illustrating that the blaster can perform at a safe distance of around 5.2 m (17 feet) and inoculate a harvester in about 5 min, this preliminary field study investigated the application of *Td/Mp* as a dry product onto a wet surface (Figure 6). We were only given access to one harvester for this trial. In this portion of the study, the harvester followed standard operating procedures in the vineyard, followed by spraying of the dry *Td/Mp* onto all surfaces that contact fruit daily. Weekly samples were acquired from the first fruit harvested by the machine at the commencement of a shift, and qPCR was performed for microbial determination and quantification. The first application of *Td/Mp* was performed once the machine possessed cells responsible for wine spoilage at a concentration of 1×10^4 cells/mL. The incorporation of *Td/Mp* into the standard sanitation protocol was measured using qPCR. There are no replicate data for this experiment, as it is anecdotal and preliminary.

Standard Operating Procedure:
• Run the harvester throughout the night, then wash the harvester down with water at ~0600 hr after finishing.
• The next evening, ~0000 hr, resume harvesting operations after wet down.

Sampling Procedure:
• Take samples from the juice and berries of the first three bins picked for the evening once they arrive on the crush pad. Send samples to ETS Laboratories for qPCR.
• Once samples have a verifiable population of 10^4, commence with the experimental operating procedure.

Experimental Operating Procedure:
• Apply *Td/Mp* as a dry product using the blaster prototype and product coverage calculations and protocol.
• Using the blaster prototype, spray the harvester with the *Td/Mp* product after finishing the standard operating procedure.

Data analysis. We received our DNA reports from ETS Laboratories. Once we transcribed the data to Microsoft Excel spreadsheets, we imported the data into the R programming environment (R Studio version 1.2.1335 Build 1379). The dependent libraries used to write and construct the models include dplyr, tidyselect, ggplot2, lsmeans, grid, rcompanion, and others. All the code generated during our data-mining expedition is listed as part of an open-source group of projects available for learning and free distribution among those interested in applied wine data science. The code used for this analysis, our statistical findings, and the data set for the project can be found at https://github.com/dandeelyon/NonSac.

Table 4 Final wine chemistry from bioreactor fermentations at UC Davis Research Winery. *Td/Mp*, a blend of *Torulaspora delbrueckii* and *Metschnikowia pulcherrima*.

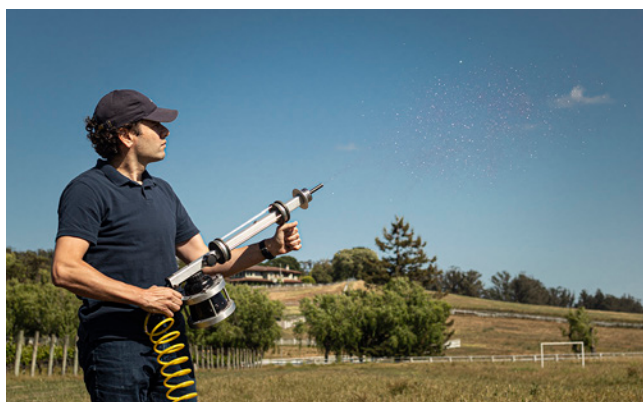| Analysis	SO_2	*Td/Mp*
Free SO_2	<2 mg/L	<2 mg/L
Volatile acidity (acetic)	0.50 g/L	0.50 g/L
L-Malic acid	<0.05 g/L	<0.05 g/L
Glucose + fructose	<0.1 g/L	0.1 g/L
Ethanol at 20°C	14.13% vol	14.05% vol
Ethanol at 60°F	14.08% vol	14.00% vol

Figure 6 Blaster shoots active dry yeasts ~5.2 m (17 ft). The yeasts adhere to minimally wet surfaces (i.e., morning dew) and develop a film as a bioprotectant.

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