Microbial Stability and Control:

EnartisStab Micro (Chitosan) Application during Wine Maturation

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2/25/2019

Webinar Formalities

- 40 min presentation
- 15 – 20 min Q&A
- Please leave questions for designated Q&A at end of the presentation, please avoid using the chat box
- Whitney is here to help
- If you are bothered by the chat box, you can toggle it closed
- We recommend using Chrome web browser. We heard negative feedback regarding Microsoft Edge
- If you are having audio issues, you can call in. Details in the chat panel
- Recorded session!

ZENITH (KPA) COLD STABILITY WEBINAR THIS FRIDAY, FEBRUARY 28th
APPROVED BY THE TTB § 24.250

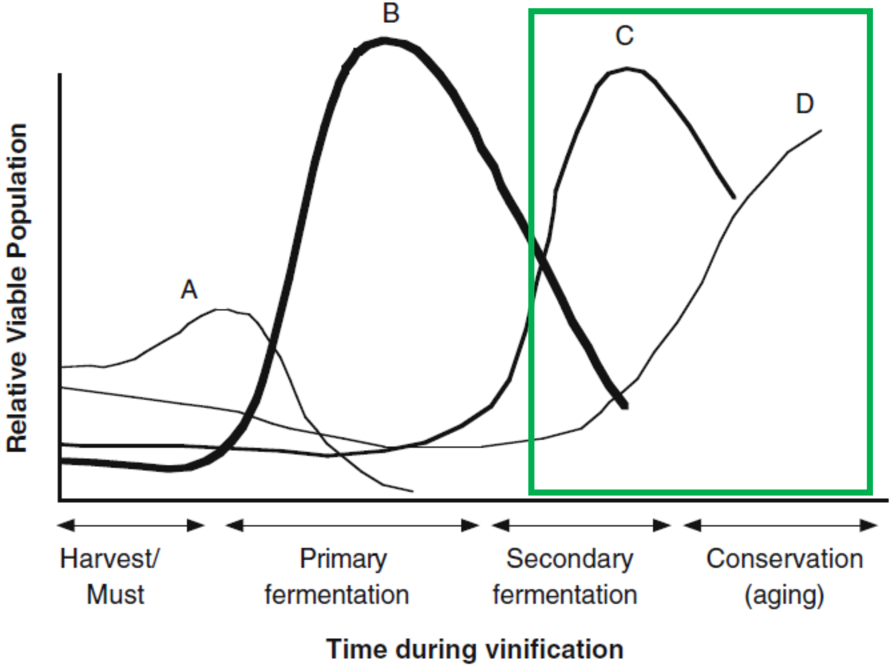
Outline

- Microbial Control
- Enological Application of Chitosan
- Antimicrobial Activity of Chitosan
- EnartisStab Micro M and EnartisStab Micro
- Winemaking Stage and Application of EnartisStab Micro
- Enartis Trial Results
- Q&A
- Citations

Wine Microbial Spoilage During Maturation

- *Malolactic Bacteria*
  - *Lactobacillus*
  - *Pediococcus*
- *Yeast*
  - *Brettanomyces bruxellensis*
  - *Zygosaccharomyces bailii*
  - *Saccharomycodes ludwigii*
- Acetic acid bacteria
  - *Acetobacter*
  - *Gluconobacter*

Microbial Ecology During Vinification



General growth of **(A) non-*Saccharomyces***, **(B) *Saccharomyces***, **(C) *Oenococcus oeni*** and **(D) spoilage yeast and bacteria** during vinification. Extracted from (Fugelsang and Edwards, 2006)

Sulfur Dioxide SO_2

- The persistent use of SO_2, has encouraged select spoilage yeast resistance (Divol et al, 2012)
  - *B. bruxellensis*
  - *Saccharomycodes ludwigii*
  - *Z. bailii (Escott et al, 2017)*

- *B. bruxellensis* demonstrates a strong niche adaptation to SO_2
  - Increased incidence of resistant strains (Avramova et al, 2019)

- *B. bruxellensis* can enter a viable-non-culturable (VBNC) state
  - Metabolism is maintained in VBNC state
  - Production and release of volatile phenols

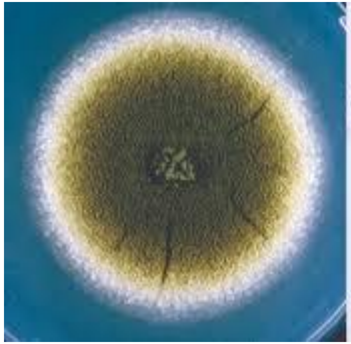
Other Microbial Control Tools

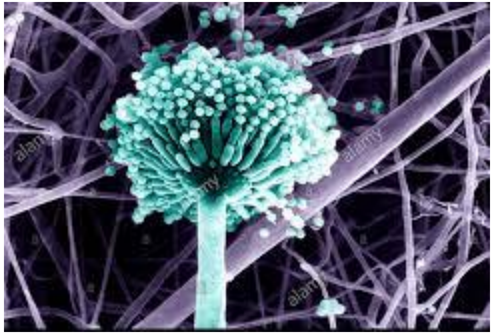
- Approved Additives:
  - Lysozyme
  - Sorbic acid
  - Fumaric acid
  - DMDC (dimethyldicarbonate)
  - Chitosan

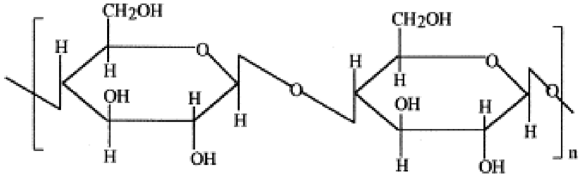
- Technologies:
  - Hydrostatic pressure
  - Ultrasonics
  - Microwave
  - Pasteurization
  - Pulsed light irradiation

What is Chitosan?

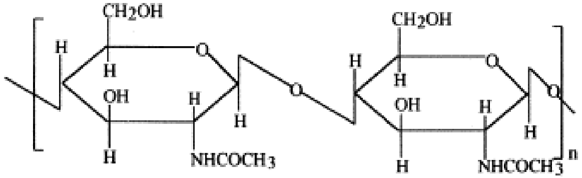
- Hydrophilic, biocompatible polymer derived from de-acetylated Chitin.
- Sourced from the mycelium/biomass of *Aspergillus niger*.
- Applications in food science, waste water treatment, agriculture and the medical industry.



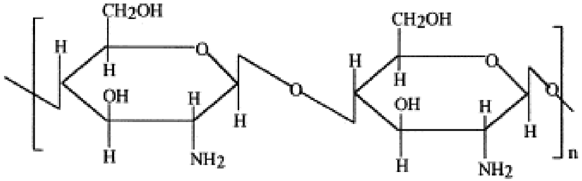




Cellulose



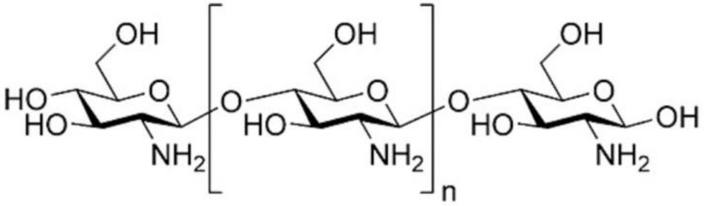
Chitin



Chitosan

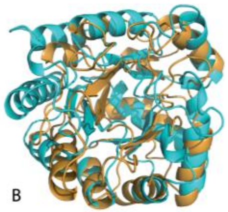
Chitosan's Enological Application

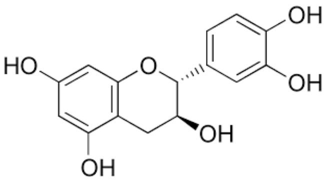
- **Reducing microbial loads:**
  - OIV & TTB (§ 24.250) 10 g/hL
- **Fining:**
  - OIV 100 g/hL
  - Chitin-glucan TTB (§ 24.250) 500g/hL
- **Ochratoxine A:**
  - OIV 500 g/hL
- **Iron, lead, cadmium and copper:**
  - OIV 100 g/hL

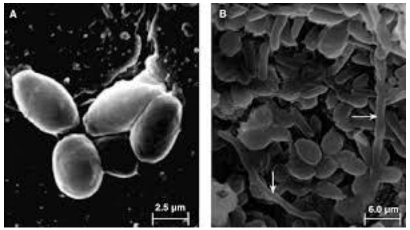


Properties of Chitosan (β-1,4-d-glucosamine)

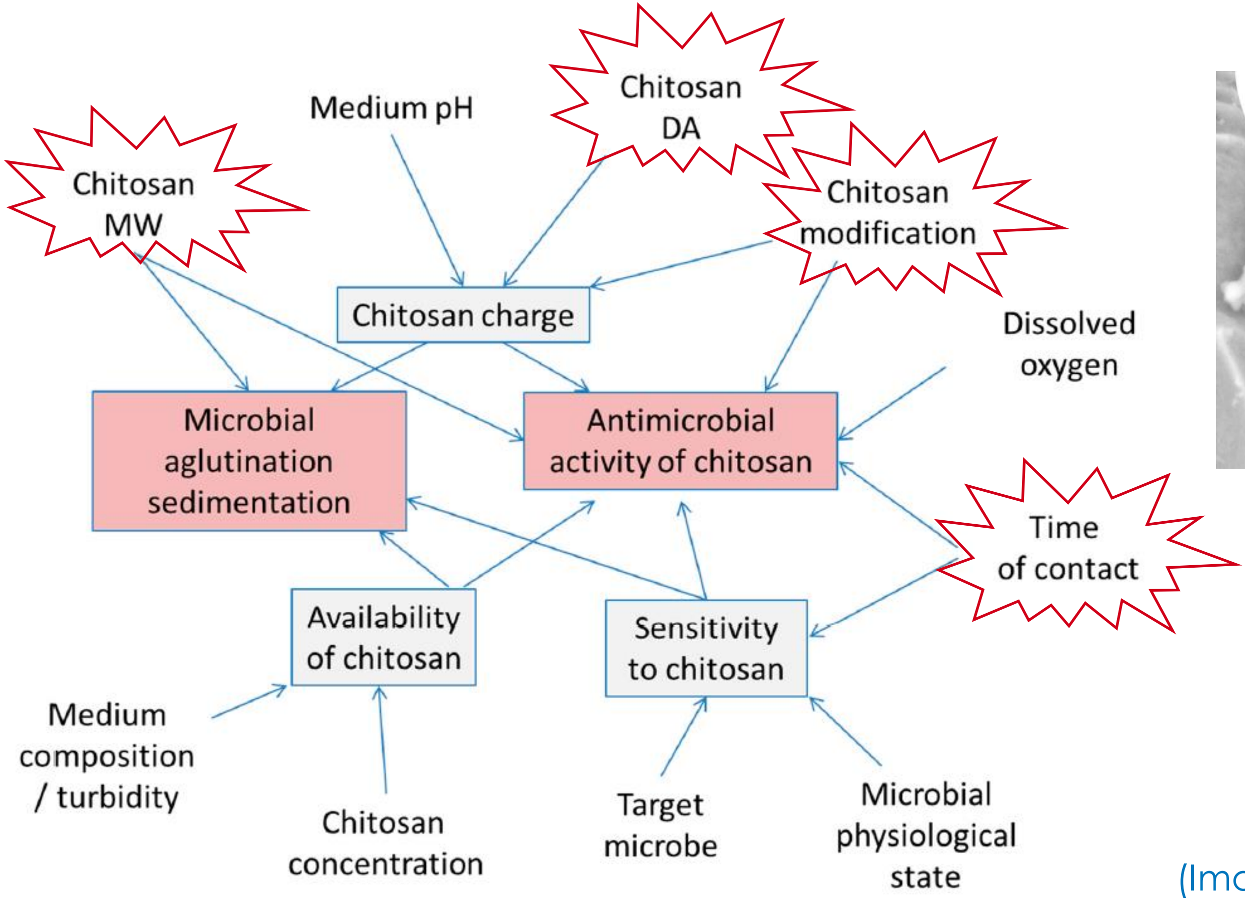
- High density of reactive amino groups and hydroxyl groups lend to its bioactivity.
- Its degree of acetylation (DD) and molecular weight (MW) determine its functional property and bioactive attributes.
- Lower MW and higher DD chitosan have increased antimicrobial properties.
- At wine pH, chitosan has a positive charge reacting with negatively charged components in wine.







Antimicrobial Activity Of Chitosan

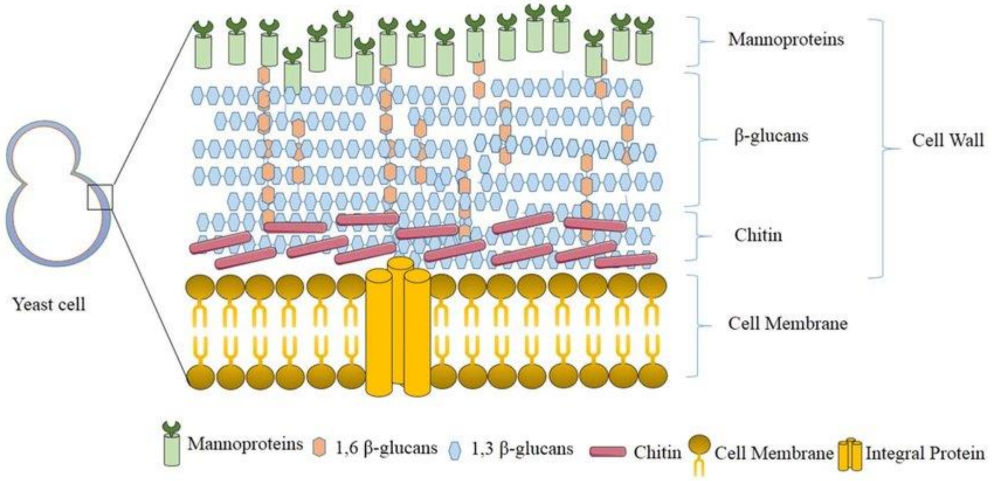


(Image from Brasselet et al, 2019)

Antimicrobial Activity Of Chitosan

Different molecular processes that may contribute to the antimicrobial activity of chitosan

- Interaction with negatively charged cell wall or cell wall components.
- Alterations to membrane permeability and energy pathways.
- Cell aggregation and physical removal through fining.
- Possible diffusion of low MW fractions through the cell wall and membrane.
- Negatively charged nutrient secretion.

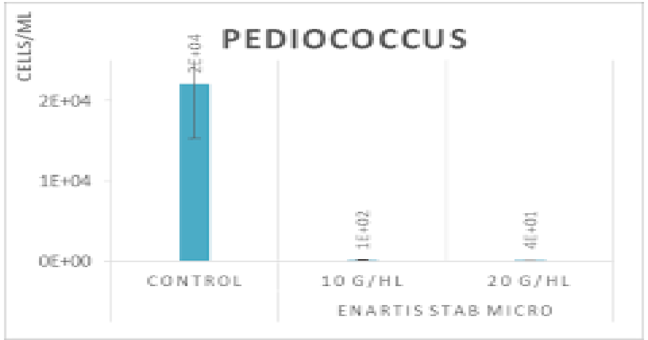


(Image from Anwar, et al 2017)

Antimicrobial Activity Of Chitosan

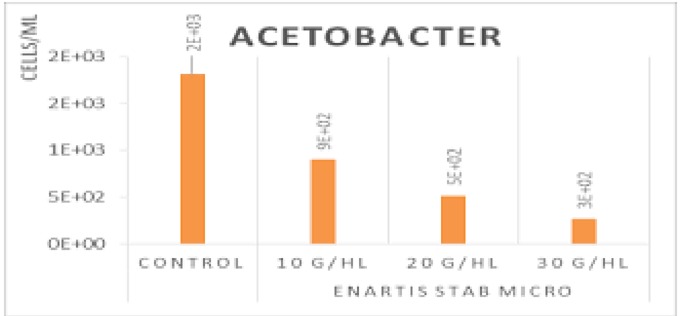
Gram-positive bacteria

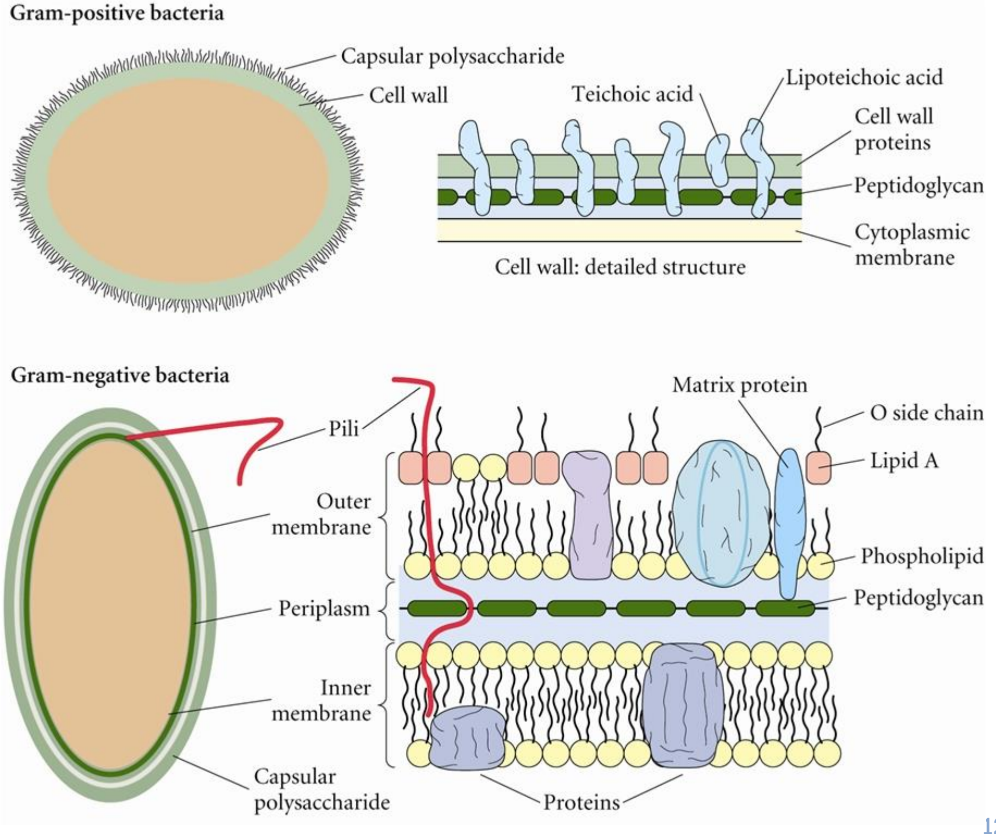
- *Oenococcus, Pediococcus,* and *Lactobacillus*



Gram-negative bacteria

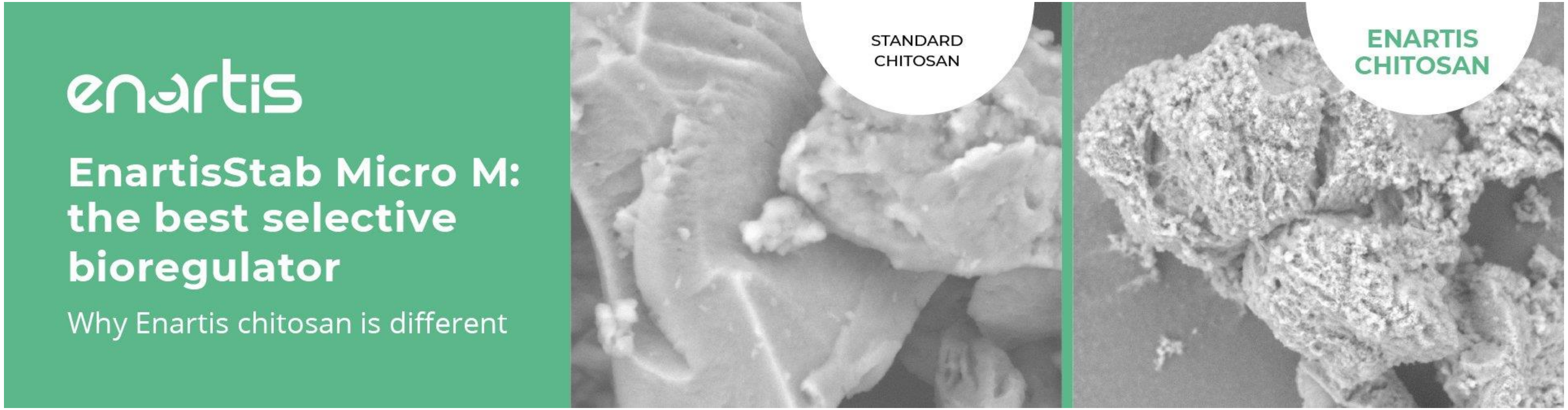
- *Acetobacter* and *Glucanobacter*





EnartisStab Micro vs. Other Chitosan Based Products

- The only enological chitosan with acid pre-activation step
- Increased reactivity and polymer surface area
- Increased charge
- Increase solubility
- Superior antimicrobial activity



EnartisStab Micro and EnartisStab Micro M

Preparation of chitosan (50%) produced from Aspergillus niger, purified yeast hulls (50%), with organic acids (ascorbic and lactic acid).

- Prevent VA during cold soak or grape transport: *5-20 g/hL*
- Remove spoilage microorganisms: *10-20 g/hL*
- Control malolactic fermentation: *5-10 g/hL*
- Prevent Stuck fermentation: *5-10 g/hL*
- **Vegan** and **allergen free** alternative to lysozyme and Sulfur dioxide

Maximum legal dosage of Stab Micro M in US: 20 g/hL

EnartisStab Micro Technical Data Sheet

Preparation of pre-activated chitosan from Aspergillus niger and organic acids (citric, lactic and ascorbic acid).

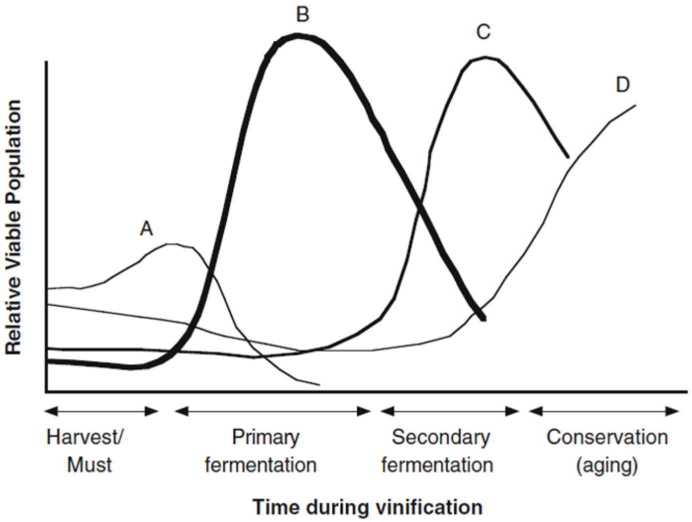
- Prevent and reduce unwanted microorganisms.
- Reduce risk of microbial growth in unfiltered bottled wines.
- Delay/avoid malolactic fermentation: an allergen free alternative to lysozyme.
- Improve clarification and filterability.
- Reduce off-flavors produced by spoilage microorganisms.

- DOSAGE: *2-15 g/hL*
  - PREVENTITIVE: *3-5 g/hL* during each transfer, racking or any wine movement.
  - CURATIVE: *10-15 g/hL*

Maximum legal dosage of Stab Micro in US: 15 g/hL

EnartisStab Micro M vs. EnartisStab Micro

Microbial Ecology During Vinification



Winemaking Phase	EnartisStab Micro M	EnartisStab Micro
Harvest Crush	X	
Flotation/Cold Settling	X	X
Fermentation	X	
Malolactic Fermentation	X	X
Barrel Aging		X
Bottling		X

General growth of (A) non-*Saccharomyces*, (B) *Saccharomyces*, (C) *Oenococcus oeni* and (D) spoilage yeast and bacteria during vinification. Extracted from (Fugelsang and Edwards, 2006)

Enartis Trial Results

Commercial Trials vs. Lab Scale

Applications of EnartisStab Micro During Maturation

- Control in laboratory vs. control in winery
- On going winery scale applications utilizing extended contact and resuspension techniques
- Inspired by multiple studies examining the impact of chitosan on *Brettanomyces* and maturation spoilage organisms during barrel ageing

vs.

vs.

(Valera et al, 2017; Nardi et al, 2014; Taillandier et al, 2015)

Experimental Design

- Objective is to prevent malolactic bacteria spoilage during maturation and preserve acidity.
- Cultures were pulled from Vinquiry Labs culture collection and acclimatized prior to inoculation:
  - B021 *Lactobacillus plantarum*
  - B001 *Oenococcus oeni*
  - B025 *Pediococcus parvulus*
- Previous studies examining effect of chitosan on *Brettanomyces* in red wine during maturation.
  - (Valera et al, 2017; Nardi et al, 2014; Taillandier et al, 2015)

Sterile filtered 2019 Alexander Valley Chardonnay

Analysis	Method	Result	Unit
Alcohol	GC	13.58	%V/V
pH	Autotitrator	3.50	
Titratable Acidity	Autotitrator	0.641	g/100mL
Free SO2	Segmented Flow	<1	mg/L
Total SO2	Segmented Flow	10	mg/L
Molecular SO2	Calculation	<0.01	mg/L
Glucose + Fructose	Enzymatic	46	mg/100mL
Malic Acid	Enzymatic	0.260	g/100mL
Lactic Acid	HPLC	0.026	g/100mL
Acetic Acid	HPLC	0.024	g/100mL

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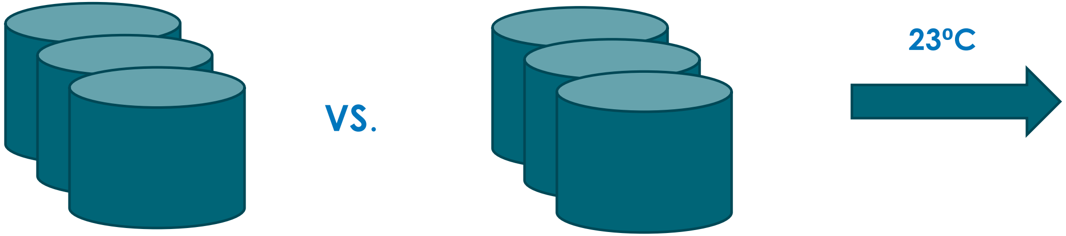
Experimental Design

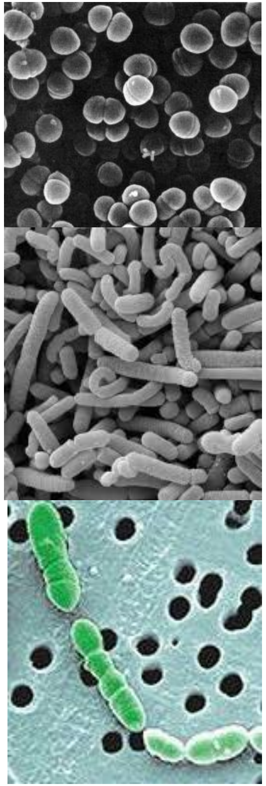
- Treatment Monitoring:
  - Treatments and controls were stirred at 400 rpm for 30 seconds, once every two weeks and sampled with EnartisStab Micro in suspension.
  - Vinquiry Lab Analysis:
    - Bi-weekly metabolite tracking: Lactic, acetic, and malic acid.
    - After 2 months: Plating/viability, CIE lab, oxidative stability, organic acids and protein stability.



- **Control:**
  - No treatment
- **EnartisStab Micro Treatment**:
  - Stab Micro dosage: 8 g/hL

VINQUIRY LABORATORIES by enartis

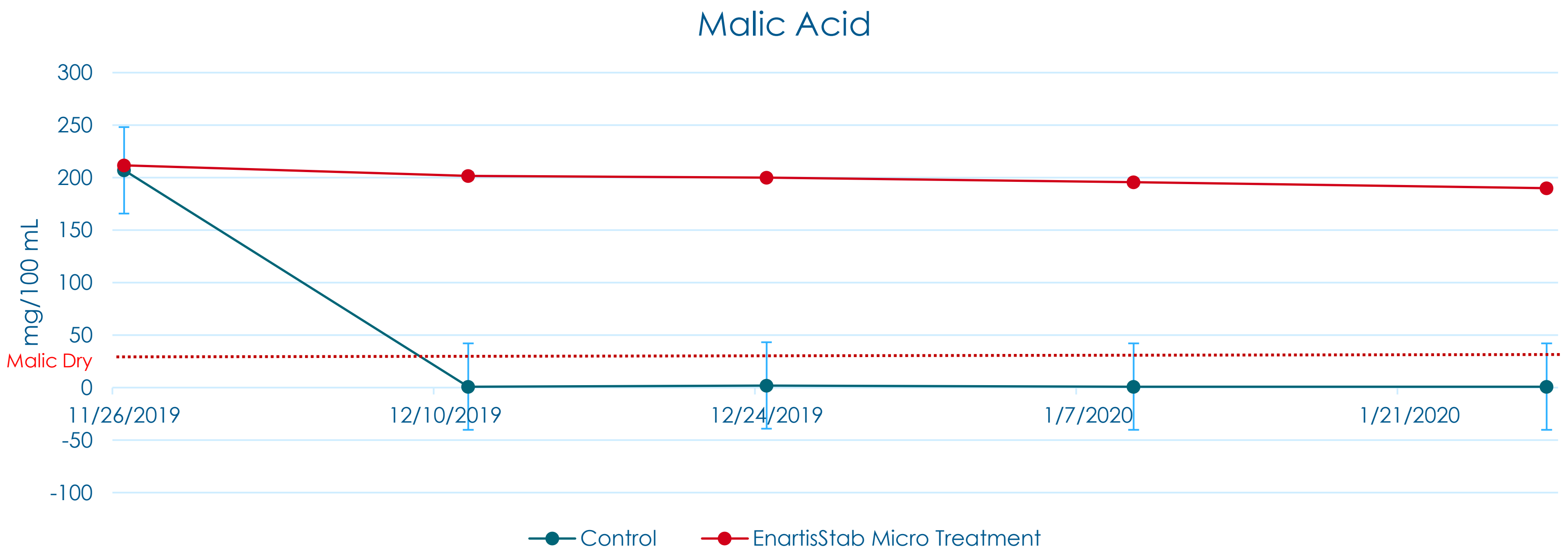
RT-PCR Initial Microbial Populations



Samples	PCR for Lactobacillaceae	PCR for Oenococcus	PCR for Pediococcus	Units
Control-1	1.88E+06	9.45E+09	3.84E+05	cells/mL
Control-2	1.61E+06	9.09E+09	2.84E+05	cells/mL
Control-3	1.43E+06	9.22E+09	2.67E+05	cells/mL
ML1a	1.18E+06	7.56E+09	2.07E+05	cells/mL
ML1b	9.85E+05	7.27E+09	1.86E+05	cells/mL
ML1C	1.15E+06	8.35E+09	2.25E+05	cells/mL

- Samples were submitted to Vinquiry Labs 12-hours after inoculation.
  - Plating results showed mixed malolactic bacteria colonies to numerous to count across all modalities.

Malic Acid Consumption

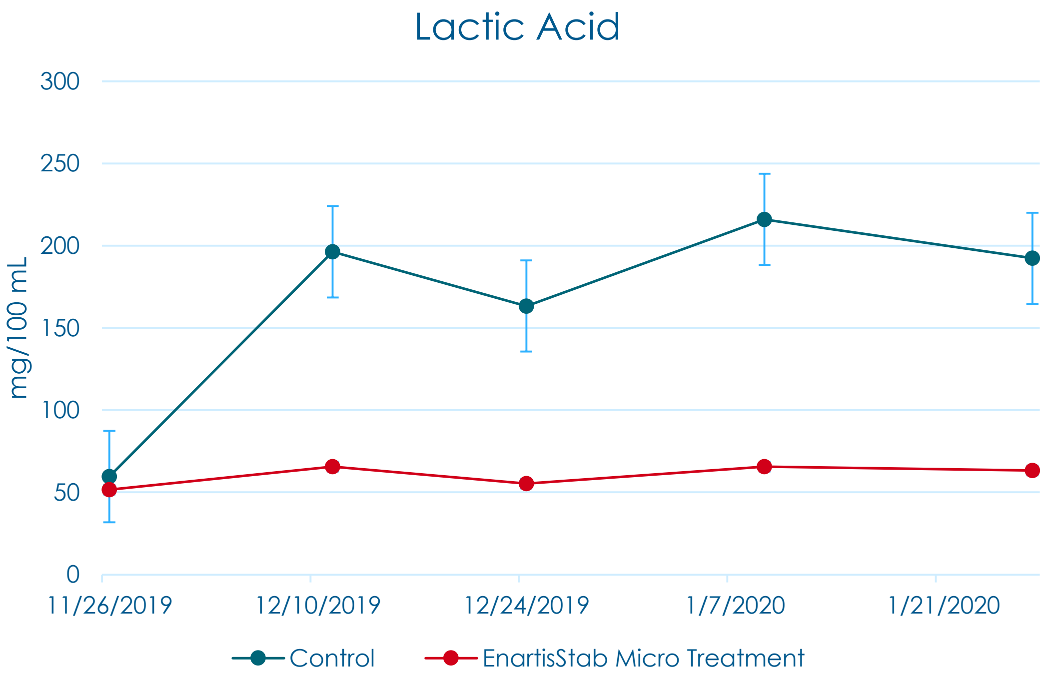


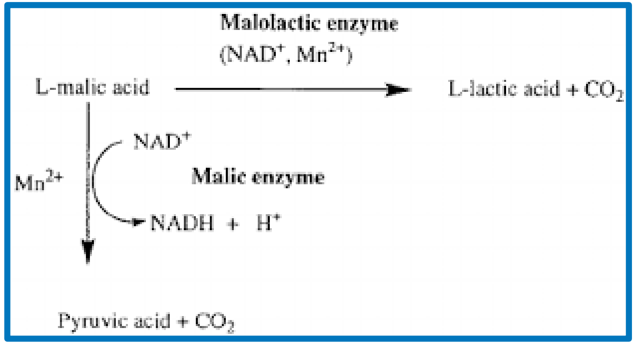
- After 3 weeks the control samples were malic dry while the EnartisStab Micro treatment maintained malic acid.

PRESERVED ACIDITY

Metabolite Data

Lactic Acid Production



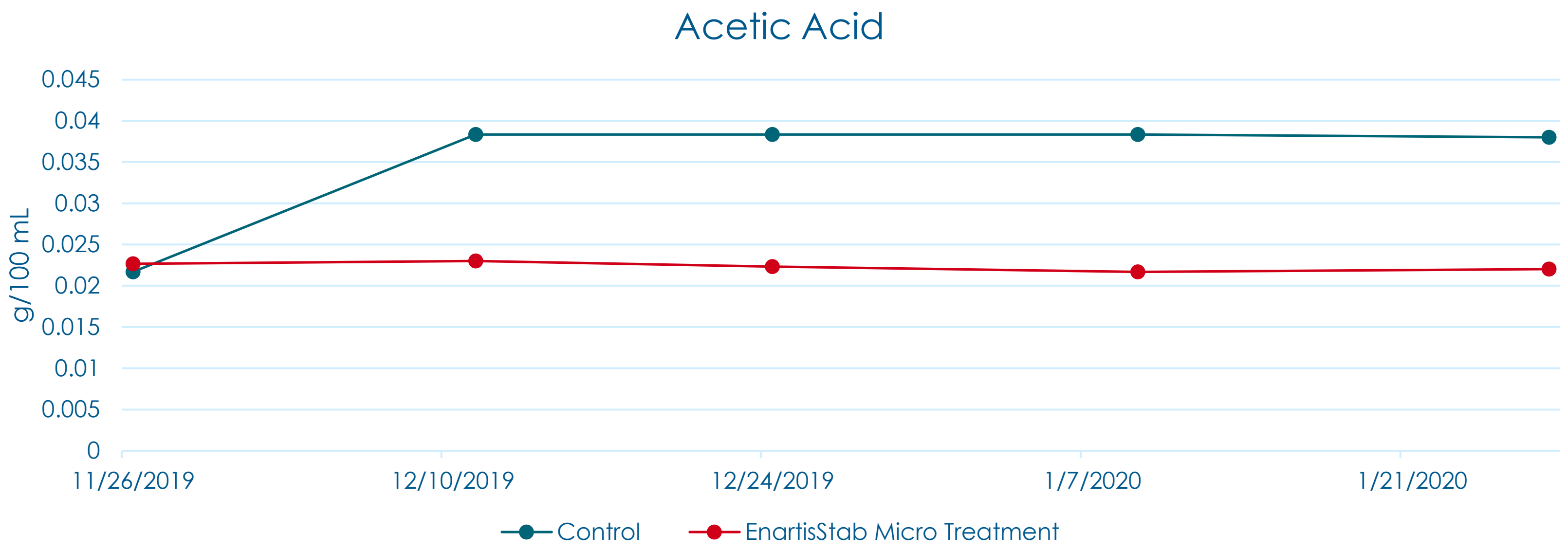


- Lactic acid significantly increased with untreated control compared to EnartisStab Micro treatment.

NO MALOLACTIC FERMENTATION

Metabolite Data

Acetic Acid Production



- Treatment with EnartisStab Micro at 8 g/hL significantly reduced the increase of acetic acid.

LOWER VOLATILE ACIDITY

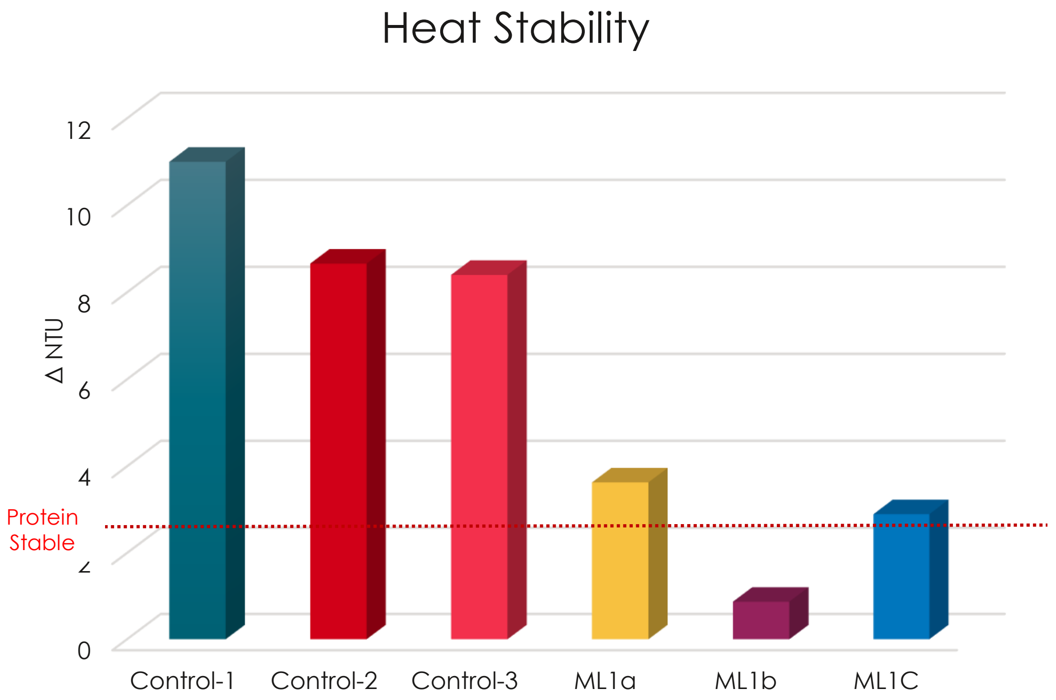
Microbial Plating

Treatments	Plating Results (CFU/mL)	Bacteria Observed
Control-1	>3.0E4	Oenococcus
Control-2	>3.0E4	Oenococcus
Control-3	>3.0E4	Oenococcus
ML1a	ND	Malolactic bacteria
ML1b	ND	Malolactic bacteria
ML1C	ND	Malolactic bacteria

- Significant effect on malolactic bacteria viability comparing EnartisStab Micro treatments vs. controls.

Additional Observations and Benefits

- Significantly increased heat stability with EnartisStab Micro treatments.
- Lower bentonite addition.
- Improved organoleptic qualities.



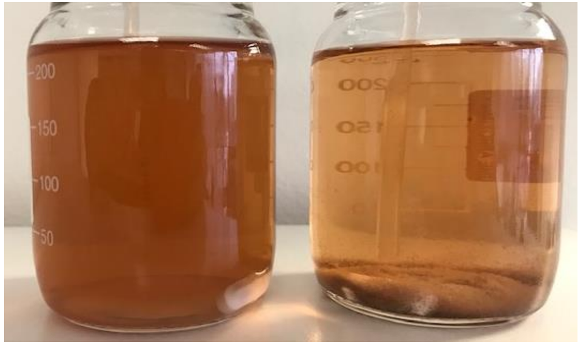
Additional Observations and Benefits

Oxidative Stability and CIE Lab Results

- Significant effect on oxidative stability and browning from EnartisStab Micro treatment vs. control.

Treatments	420 nm abs	Result
Control-1	0.174	Unstable
Control-2	0.179	Unstable
Control-3	0.176	Unstable
ML1a	0.127	**Stable**
ML1b	0.87	**Stable**
ML1c	0.144	**Stable**

(Iland et al, 1993)



Treatments	CIE Color Differences (ΔE)
Control 1 - ML1a	5.29
Control 2 - ML1b	5.80
Control 3 - ML1c	6.18

- CIE Color Differences ΔE :
  - 2.0 - 10.0 Perceptible at a glance.

Other Industrial Applications

Immunoadjuvant
Anticancer
Biodegradable
Non-toxic
Clarification
Safe
Renovated
Fungistatic
Biocompatible
Osteoblast-stimulation
Antibacterial
Food
Medical
Anti-cholesteremic
Spermicidal
Hemostatic
Bio-ethanol
Fining
Antitumor
Waste-water

What can't chitosan do!!!!

Chitosan Blended Products and self-brett™ Diagnosis Kit

Other Products

- **Claril QY:** Phenolic and off flavor fining with autolyzed yeast and chitosan
- **Claril ZR:** Vegan fining agent from plant protein, chitosan, and bentonite.
  - Preparing red wine for Zenith Color
- **Claril ZW:** Vegan fining agent from plant protein , chitosan, and sodium activated bentonite.
  - Preparing white wine for Zenith Uno
- **Claril HM:** PVIPVP and pre-activated Chitosan for heavy metal removal and prevention of browning/oxidation.

Fining and Clarification Webinar Summer 2020

Enartis 2020 Handbook download: https://www.enartis.com/wp-content/uploads/2019/04/Enartis-Handbook-2020.pdf

- **self-brett™:** A compact diagnosis kit that allows winemakers to analyze for Brettanomyces independently of a laboratory or more expensive analytical techniques.
  - Anecdotal, affordable diagnostic tool without the requirement of specialized equipment.
  - Price: $20.00
  - Turnaround time: 7-10 days

15 minute Q&A

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Citations

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