UNIVERSIDADE DE CAXIAS DO SUL
ÁREA DE CONHECIMENTO DE CIÊNCIAS DA VIDA
INSTITUTO DE BIOTECNOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA

Estresse, comportamento das leveduras durante a segunda fermentação e diferenças entre os métodos Tradicional e Charmat na elaboração de espumantes

BRUNO CISILOTTO

UCS
Caxias do Sul, RS – Brasil
2023

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Tese apresentada ao Programa de Pós-graduação em Biotecnologia da Universidade de Caxias do Sul, como parte dos requisitos para a obtenção de grau de Doutor em Biotecnologia.

Orientador: Prof. Dr. Sergio Echeverrigaray
Coorientador: Prof. Dr. Tomás Román Villegas

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Dedico este trabalho a todos os pesquisadores da área de enologia e disciplinas relacionadas que se empenham a aperfeiçoar os seus conhecimentos e contribuir com o desenvolvimento da vitivinicultura brasileira.

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“Maravilhar-se é o primeiro passo para um descobrimento.”

Louis Pasteur

Resumo

Estudos sobre os processos envolvidos na elaboração de espumantes podem contribuir para o avanço dos conhecimentos científicos em enologia e biotecnologia. Neste contexto biotecnológico, a levedura *Saccharomyces cerevisiae* desempenha um papel importante. O tema central desta tese é a elaboração de espumantes por meio de duas fermentações e o comportamento das leveduras durante esse processo. O trabalho está dividido em três capítulos e seis apêndices, nos quais apresentamos os estudos realizados durante o curso de Doutorado em Biotecnologia. No capítulo 1, foram realizados testes em diferentes ambientesfermentativos, com e sem a presença de etanol e SO_2, simulando uma segunda fermentação paraa produção de espumantes. Demonstramos que o estresse causado pela interação entre o SO_2 eo etanol presentes no vinho base reduz consideravelmente a viabilidade das leveduras no iníciodesse processo, podendo ser considerado o principal inibidor das leveduras nessa etapa. Nocapítulo 2, comparamos os métodos de elaboração de espumantes Tradicional e Charmat.Mostramos que a homogeneização contínua do vinho após o término da segunda fermentação,durante o envelhecimento dos espumantes no método Charmat, antecipa os processosautofágicos das leveduras, acelerando a morte celular em comparação com o método Tradicional, no qual as leveduras envelhecem juntas no fundo da garrafa. No capítulo 3, pormeio de análises químicas e sensoriais, demonstramos que os espumantes elaborados pelosmétodos Tradicional e Charmat, a partir do mesmo vinho base, inóculo e tempo deenvelhecimento, apresentam características olfativas e gustativas muito similares. Concluímosque o método em si não determina a qualidade dos produtos. Nos apêndices, são apresentados,em ordem cronológica, um trabalho apresentado em um evento, duas publicações de artigostécnicos relacionados ao capítulo 1 e três resumos enviados a congressos, abordando estudossobre a modelagem aromática de espumantes usando diferentes cepas de leveduras, tipos denutrientes e a caracterização aromática de espumantes elaborados com variedades de uvasresistentes a doenças. Os resultados obtidos nesta tese e as conclusões elaboradas podemcontribuir para o avanço do conhecimento na elaboração de espumantes e, indiretamente,auxiliar no desenvolvimento da vitivinicultura nacional.

Palavras-chave: *Saccharomyces cerevisiae*; SO_2; viabilidade das leveduras; processos autofágicos; morte celular; características organolépticas.

Abstract

Studies on the processes involved in the production of sparkling wines can contribute to the advancement of scientific knowledge in oenology and biotechnology. In this biotechnological context, the yeast *Saccharomyces cerevisiae* plays an important role. The central theme of this thesis is the production of sparkling wines through two fermentations and the behaviour of yeasts during this process. The work is divided into three chapters and six appendices, in which we present the studies conducted during the Doctoral course in Biotechnology. In Chapter 1,tests were conducted in different fermentation environments, with and without the presence ofethanol and SO_2, simulating a second fermentation for sparkling wine production. Wedemonstrated that the stress caused by the interaction between SO_2 and ethanol present in thebase wine considerably reduces the viability of yeasts at the beginning of this process, whichcan be considered the main yeast inhibitor in this stage. In Chapter 2, we compared theTraditional and Charmat methods of sparkling wine production. We showed that the continuoushomogenization of the wine after the completion of the second fermentation, during the agingof sparkling wines using the Charmat method, triggers the autophagic processes of yeasts,accelerating cellular death compared to the Traditional method, in which yeasts age together atthe bottom of the bottle. In Chapter 3, through chemical and sensory analyses, we demonstratedthat sparkling wines produced by the Traditional and Charmat methods, using the same basewine, inoculum, and aging time, exhibit very similar olfactory and gustatory characteristics. Weconcluded that the method itself does not determine the quality of the products. In theappendices, a work presented at an event, two publications of technical articles related toChapter 1, and three abstracts submitted to conferences are presented in chronological order.These address studies on the aromatic modelling of sparkling wines using different yeast strains,nutrient types, and the aromatic characterization of sparkling wines made from disease-resistantgrape varieties. The results obtained in this thesis and the conclusions drawn can contribute tothe advancement of knowledge in sparkling wine production and indirectly assist in thedevelopment of national vitiviniculture.

Keywords: *Saccharomyces cerevisiae*; SO_2; yeast viability; autophagic processes; cell death; organoleptic characteristics.

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Sumário

1 Introdução

O Brasil destaca-se pela sua notável habilidade na produção de espumantes de alta qualidade e autenticidade, principalmente na região da Serra Gaúcha. Esses produtos são amplamente reconhecidos nacional e internacionalmente (Araujo et al., 2019; Wurz et al., 2017). Além de serem considerados de boa qualidade, os espumantes brasileiros possuem características distintas que lhes conferem destaque, resultando em um aumento significativo na demanda e gerando retornos financeiros substanciais para as empresas vinícolas (Pereira et al., 2020).

A partir de um vinho base seco (sem açúcares residuais fermentescíveis), dois métodos de produção são os mais utilizados na elaboração de espumantes: o método Tradicional, no qual a segunda fermentação ou "tomada de espuma" ocorre em garrafas de vidro vedadas, e o método "Charmat", onde a segunda fermentação é ocorre em tanques de pressão (Moreno-Arribas and Polo, 2009). Ambos os métodos envolvem a utilização de um vinho base, a adição de açúcar, nutrientes e leveduras, possibilitando o desenvolvimento de uma nova fermentação, denominada segunda fermentação, na qual o gás carbônico produzido permanece dissolvido no produto, gerando a "espuma", considerada uma das principais características destes produtos (Liger-Belair, 2017; Moreno-Arribas and Polo, 2009).

Uma das etapas mais sensíveis e que necessita de cuidados nesse processo é o início da segunda fermentação, onde as leveduras que vão realizar a tomada de espuma irão entrar em contato com um novo meio fermentativo considerado inóspito, pois, no vinho base há alta concentração de etanol, presença de glicerol e pH baixo (Borrull et al., 2015), presença de dióxido de enxofre (SO_2) (Sudraud et al., 1985), presença de ácido acético (Giannattasio et al., 2013), temperaturas relativamente baixas de fermentação e baixa concentração de nutrientes (Kemp et al., 2020; Martí-Raga et al., 2015). Todos esses fatores, individualmente e em conjunto, causam estresse nas leveduras.

Nos espumantes elaborados pelo método Tradicional normalmente a maturação em contato com as borras dura um período maior ou igual a nove meses (dependendo da região de produção). Durante este período, quando não há mais fonte de nutrientes disponível, as leveduras podem entrar em processos autofágicos, passando pelo processo de autólise, liberando compostos como manoproteínas, glucanos e outros componentes intracelulares que aumentam a complexidade do vinho, se tornando assim uma espécie de moduladores das características sensoriais gustativas (Alexandre and Guilloux-Benatier, 2006). Por outro lado,

os espumantes produzidos pelo método Charmat tendem a permanecer períodos menores em contato com as "borras", normalmente inferiores a seis meses, sendo característica deste tipo de produto apresentar aromas mais frescos e frutados de um vinho jovem (Moreno-Arribas and Polo, 2009; Togores, 2018). No entanto, existe a possibilidade do contato entre borras e o vinho se estender por um período maior também no método Charmat, ocorrendo maior liberação dos componentes da levedura para o vinho, além da evolução do vinho por processos oxido-redutivos. Esse tempo pode variar de acordo com o resultado que se pretende obter (Jackson, 2020).

Uma diferença entre os dois métodos de elaboração dos espumantes ocorre durante o processo de envelhecimento do vinho em contato com as leveduras, em que, no método Tradicional, normalmente as borras permanecem no fundo da garrafa (posição horizontal). Como a garrafa é considerada um recipiente pequeno, teoricamente haveria uma maior interação das borras com o vinho. Porém, no método Charmat, no caso de os tanques de pressão possuírem agitadores internos, as borras podem estar em suspensão intermitentemente deixando o vinho homogêneo (Jackson, 2020; Ribéreau-Gayon et al., 2021; Togores, 2018), ocorrendo assim um contato ainda maior do vinho com as leveduras.

Os efeito positivos nas características organolépticas causada pela autólise das leveduras é conhecida (Di Gianvito et al., 2019). A autólise (Alexandre, 2011; Alexandre and Guilloux-Benatier, 2006; Torresi et al., 2011), autofagia e apoptose (Cebollero et al., 2005; Piggott et al., 2011; Preiss et al., 2018; Reggiori and Klionsky, 2013; Rego et al., 2013; Yousefi and Simon, 2007) em leveduras, são fenômenos que tem sido amplamente estudados. Estes processos (autofagia, autólise) estão associados e são propiciados por deficiência nutricional entre outras condições de estresse, comuns no ambiente enológico (Cebollero and Gonzalez, 2006). A autólise ocorre durante o envelhecimento dos vinhos espumantes nos quais as leveduras são submetidas a baixa disponibilidade de nitrogênio e carbono (Leroy et al., 1990; Moreno-Arribas et al., 2000). A autofagia tem sido relatada durante o envelhecimento de vinhos espumantes e em outros vinhos, sendo caracterizada pelas mudanças morfológicas e formação de corpos autofágicos (Cebollero et al., 2005), fator que deve ser levado em consideração na contribuição sensorial do envelhecimento dos vinhos nas borras.

A sequência de eventos que levam até a autólise das leveduras é consideravelmente conhecida em vinhos, principalmente com estudos realizados em espumantes elaborados pelo método Tradicional (Babayan and Bezrukov, 1985; Fornairon-Bonnefond et al., 2002; Gnoinski et al., 2021b; A. J. Martínez-Rodríguez et al., 2001), entretanto estes eventos não têm sido estudados nas situações peculiares estabelecidas pelo método Charmat. Além disso, faltam

trabalhos sobre o estresse nas leveduras durante a inoculação, no início da segunda fermentação, fator que pode comprometer ou dificultar a realização da tomada de espuma. Uma outra curiosidade entre os métodos de elaboração, segundo diversos autores (Blouin and Peynaud, 2006; Ribéreau-Gayon et al., 2021; Togores, 2018), é que na tomada de espuma para elaboração de espumantes, existe uma tendência em classificarem o método Tradicional como melhor opção no quesito qualidade e complexidade do produto, quando comparado com os espumantes obtidos pelo método Charmat. Existem alguns estudos na literatura que comparam esses dois métodos, entretanto, os trabalhos realizados até o momento avaliaram espumantes obtidos com uvas, vinhos base, cepas de leveduras e tempos de envelhecimento nas borras diferentes entre os métodos (Caliari et al., 2015; J. A. Culbert et al., 2017; Ubeda et al., 2016; Vecchio et al., 2018). Pensando nisso, partindo de um mesmo vinho base e inóculo, neste estudo foram realizadas avaliações do comportamento das leveduras no início da segunda fermentação, durante a tomada de espuma e posterior envelhecimento de espumantes elaborados em ambos métodos (Tradicional e Charmat), junto de uma avaliação da composição dos espumantes e suas características organolépticas ao longo do tempo, tendo como pretensão entender com maior profundidade o que efetivamente acontece com as leveduras e com cada tipo de produto elaborado nesses dois distintos métodos.

2 Revisão Bibliográfica

2.1 Vinhos espumantes

Vinhos espumantes são produzidos em diversas regiões vitivinícolas ao redor do mundo utilizando diferentes cepas de uvas e estilos de vinificação (Di Gianvito et al., 2019). Além disso, pode ocorrer que a legislação específica de cada país ou região produtora regule as matérias-primas, alguns parâmetros físico-químicos do vinho base e as tecnologias e processos de vinificação para garantir um padrão de qualidade desses vinhos (Pozo-Bayón et al., 2009). Entretanto, todos os espumantes apresentam características em comum, sendo uma das principais, a origem do gás carbônico endógeno proveniente da fermentação alcoólica de um mosto ou da segunda fermentação de um vinho base de espumante (Di Gianvito et al., 2019; Togores, 2018).

Segundo o artigo 11 da Lei n°10.970 de 12 de novembro de 2004, conhecida como a Lei do Vinho**,** a definição de vinho espumante no Brasil é:

> Art. 11. Champanha (Champagne), Espumante ou Espumante Natural é o vinho cujo anidrido carbônico provém exclusivamente de uma segunda fermentação alcoólica do vinho em garrafas (método Champenoise/Tradicional) ou em grandes recipientes (método Chaussepied/Charmat), com uma pressão mínima de 4 (quatro) atmosferas a 20 ºC (vinte graus Célsius) e com teor alcoólico de 10 % (dez por cento) a 13 % (treze por cento) em volume (BRASIL, 2004).

De um ponto de vista estritamente físico-químico, vinhos espumantes são sistemas hidroalcóolicos dotados de diversos componentes, com uma densidade próxima da água e uma viscosidade de cerca de 50 % maior que a da água pura (principalmente devido à presença de aproximadamente 12,5 % de etanol). É um produto supersaturado, com CO_2 dissolvido, formado juntamente com o etanol durante o processo de tomada de espuma (segunda fermentação sob pressão em tanques ou garrafas). Essa fermentação ocorre devido a adição de uma quantidade específica de açúcares por litro de vinho e leveduras que realizam a segunda fermentação. Uma garrafa padrão de 750 ml contém cerca de 9 g de CO_2, que corresponde a um volume próximo a 5 litros de CO_2 gasoso sob condições padrão de temperatura e pressão (Liger-Belair, 2016).

O fato da fermentação do espumante ocorrer em um ambiente hermético permite o aumento da pressão e incorporação do gás carbônico ao líquido por meio da fermentação alcoólica ($C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2$). Entre 16 e 18 g/L de açúcar fermentado, forma-

se aproximadamente 1 % de álcool (Ribéreau-Gayon et al., 2021). A cada grama de açúcar/litro consumido é produzido 0,46 g de gás carbônico/litro, e a cada 4 gramas de açúcar/litro consumido é gerada aproximadamente 1 atm de pressão a 20 °C (Degré, Ortiz-Julien, Wardrop, & Zhang, 2019).

Existem dois métodos principais de elaboração de espumante a partir de um vinho base: o método *Granvas*, *Bulk*, *Charmat*, onde a segunda fermentação do vinho base ocorre em um tanque isobárico e o método *Champenoise*, *Tradicional* ou *Clássico*, no qual a fermentação do vinho base ocorre em uma garrafa selada (Buxaderas and López-Tamames, 2010). Atualmente, segundo os regulamentos da União Europeia (EEC N° 3309/85 de 18 de novembro de 1985 e EEC N° 2333/92 de 13 de julho de 1992) os termos *"champagne" e* "*méthode champenoise*" referindo-se ao espumantes elaborados pelo método Tradicional, só podem ser oficialmente utilizados para os vinhos espumantes produzidos dentro da denominação de origem de *Champagne* na França. Neste trabalho foi adotado somente os termos "método Charmat" e método Tradicional" pois são os dois termos mais amplamente utilizados para descrever estes dois métodos de tomada de espuma no Brasil (Gabbardo and Celotti, 2015; Guerra et al., 2019; Nierdele et al., 2016).

Diferente de um vinho tranquilo em que a fermentação pode ocorrer de forma espontânea, nos vinhos espumantes de duas fermentações não há essa possibilidade. Desse modo, existem duas fases principais na elaboração desses espumantes. A primeira é a fermentação do mosto de uva para convertê-lo em um vinho base de espumante (é um "vinho seco", ou seja, sem resíduos de açúcares fermentescíveis). Já a segunda etapa ocorre após a adição de açúcares e leveduras (licor de tiragem - *liqueur de tirage)* no vinho base que passa por uma segunda fermentação (chamada de segunda fermentação ou tomada de espuma - *prise de mousse*) no tanque de pressão ou na garrafa (Kemp et al., 2015; Ribéreau-Gayon et al., 2021; Togores, 2018).

Na elaboração de espumantes com duas fermentações, é imprescindível a utilização de leveduras selecionadas e adaptadas, pois já existe uma concentração de etanol relativamente alta, pH baixo e baixa disponibilidade de nutrientes (Borrull et al., 2016; Martí-Raga et al., 2015). A inoculação normalmente ocorre com concentrações de 1 a 2.10^6 células por ml. Concentrações mais baixas conduzem a fermentações lentas, podendo ocorrer sobras residuais de açúcares fermentescíveis e concentrações mais altas, apesar da fermentação ocorrer mais rapidamente, pode ocasionar a formação de aromas e gostos anormais para os vinhos (Ribéreau-

Gayon et al., 2021; Togores, 2018). A Figura 1 mostra as etapas dos métodos Charmat e Tradicional, com suas similaridades e peculiaridades.

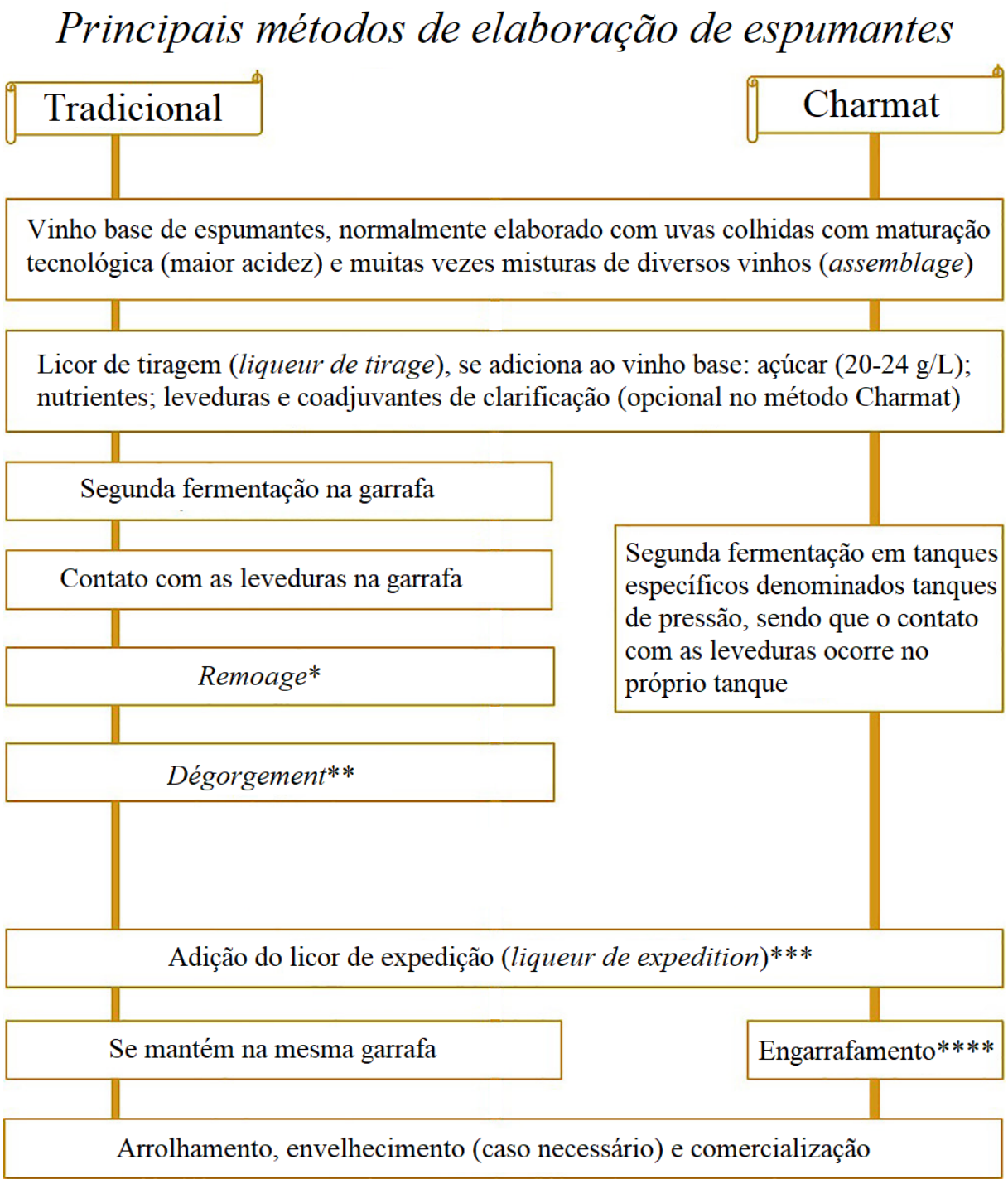

Figura 1: Diferenças e similaridades dos dois métodos mais utilizados na elaboração de espumantes com duas fermentações. * Processo pelo qual as leveduras e sedimentos (borras) contidas no vinho são movimentadas no sentido do gargalo da garrafa, sendo que ao final do processo todo o sedimento está acumulado no gargalo para posterior retirada. Esse processo normalmente tem auxílio de um coadjuvante de clarificação; ** Retirada dos sedimentos da garrafa, sendo que esse processo pode ser feito com o congelamento do bico da garrafa (*à la glace*) ou sem o congelamento (*à la volée*); *** O licor de expedição é basicamente composto de açúcar que irá definir o

“estilo” do espumante (extra brut, brut, demi-sec e outros), ácido orgânico caso haja necessidade de correção e conservantes, sendo o dióxido de enxofre (SO_2) o mais utilizado; **** Antes do engarrafamento do espumante é realizado uma filtração isobárica. Adaptado de: (Di Gianvito et al., 2019; Togores, 2018).

A Figura 2 mostra uma possibilidade de instalações com as etapas especificas para a produção de espumantes pelo método Charmat em tanques de pressão feitos normalmente de aço inoxidável.

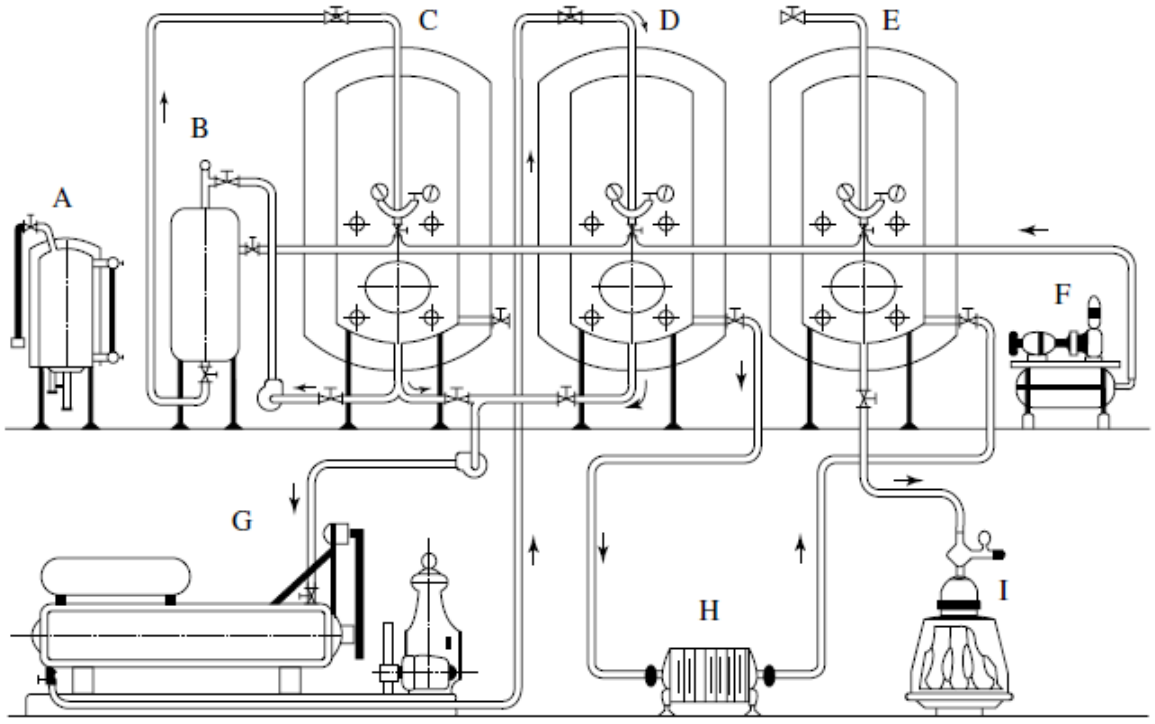

Figura 2: Exemplo de instalação para espumatização pelo método Charmat. **A**- Tanque de preparação das leveduras; **B**- tanque de adição de açúcar, dotado de um misturador; **C**- tanque para a segunda fermentação; **D**- tanque de refrigeração; **E**- tanque pulmão de engarrafamento; **F**- compressor de ar para engarrafamento isobaromético; **G**- sistema de refrigeração; **H**- filtro isobárico; **I**- enchedora (envasadora) isobárica. Adaptado de: (Ribéreau-Gayon et al., 2021).

No método Tradicional todo o processo (fermentação e envelhecimento) é realizado na mesma garrafa que entra no mercado e vai para o consumidor final (Pozo-Bayón et al., 2009). A Figura 3 mostra as etapas clássicas de produção de espumantes pelo método Tradicional.

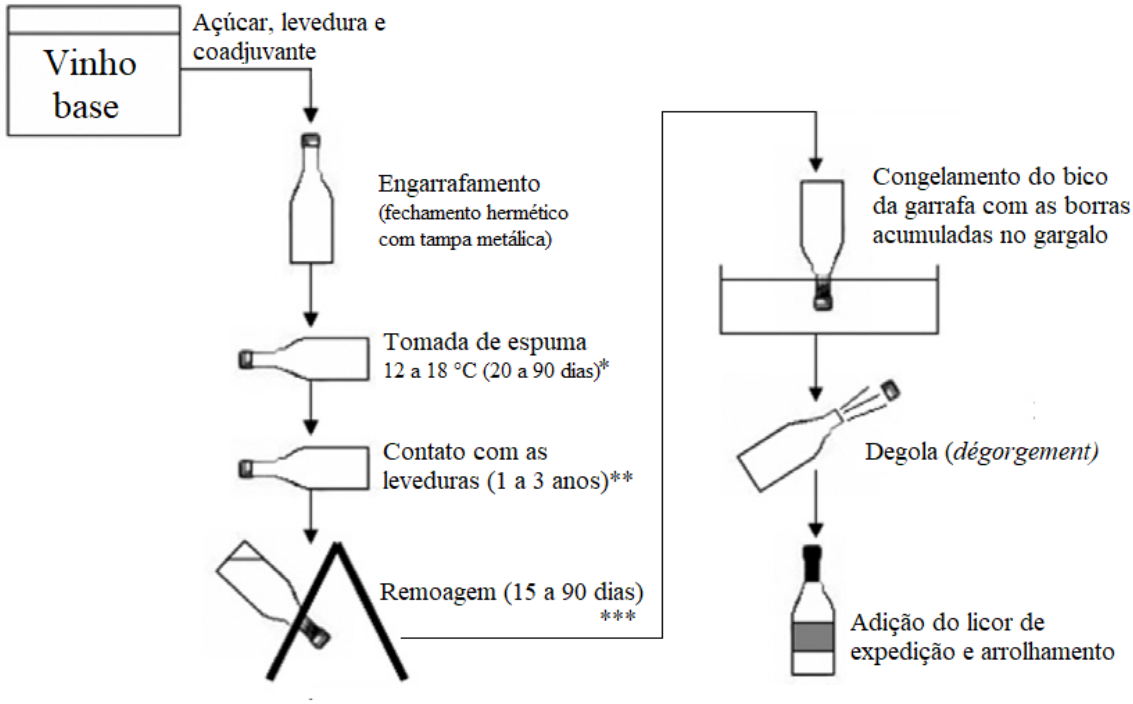

Figura 3: Exemplo das etapas de espumatização pelo método Tradicional. *O tempo e a cinética fermentativa variam muito de acordo com a temperatura de fermentação; **Esse período de contato com as leveduras pode variar bastante, podendo ser menor que 1 ano, porém também, podendo passar dos 3 anos; ***Atualmente esse processo denominado remoagem (*remuage*) pode ser feito na forma automatizada com o auxílio de um instrumento denominado "giropalete" aumentando a velocidade do processo. Adaptado de: (Colagrande et al., 1994; Torresi et al., 2011).

2.2 Leveduras Enológicas

Foi o Luis Pasteur em 1866, em seu trabalho "*Etudes sur le vin"* que estabeleceu que os mostos fermentados espontaneamente eram resultado do metabolismo de leveduras que se encontravam na película das uvas (Ribéreau-Gayon et al., 2021). Dentre as diversas espécies de leveduras existentes nas uvas, a espécie *Saccharomyces cerevisiae* é principal responsável pela fermentação alcoólica. As cepas dessa espécie possuem características tecnológicas diversas, sendo que muitas são comercializadas e rotuladas por suas características (Regodón Mateos et al., 2006; Salinas et al., 2012). São microrganismos facultativos, ou seja, eles podem sobreviver e se multiplicar em condições aeróbicas ou anaeróbicas (com ou sem oxigênio). Podem se reproduzir sexuadamente (formação de ascósporos), porém em condições enológicas e de nutrição adequada, se reproduzem normalmente de forma assexuada por gemiparidade (brotamento) (Fugelsang and Edwards, 2007; Moreno and Peinado, 2012; Ribéreau-Gayon et al., 2006a). Enquanto a sua forma, possuem aspecto subesférico e elíptico, com dimensões que

podem variar de 3 a 10 x 5 a 12 µm (Togores, 2018). A Figura 4 mostra detalhes da morfologia de uma levedura *S. cerevisiae*.

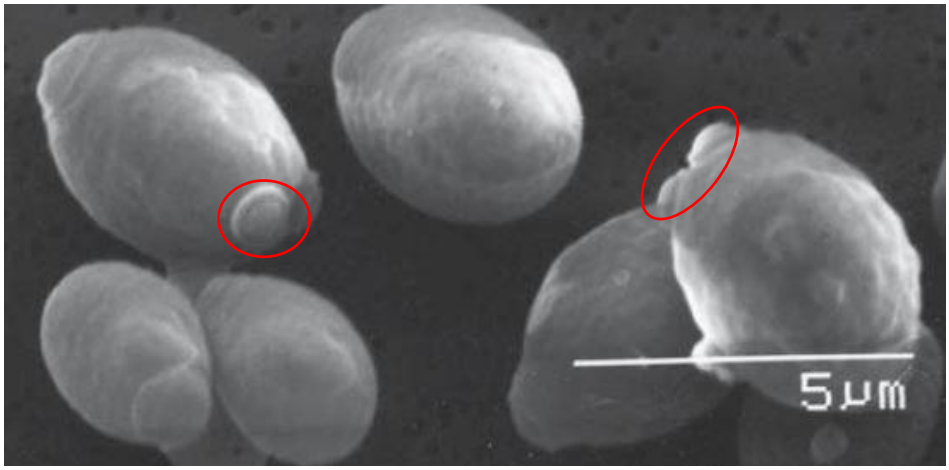

Figura 4: Detalhes de leveduras da espécie *Saccharomyces cerevisiae* onde é possível ter uma perspectiva do tamanho e visualizar as cicatrizes (círculo vermelho) ocasionadas pela reprodução por brotamento. Adaptado de: (Fugelsang and Edwards, 2007).

Como comentado anteriormente, na elaboração de espumantes, para a realização da segunda fermentação, é imprescindível a utilização de cepas de leveduras adaptadas ao meio hidroalcoólico e a baixa disponibilidade de nutrientes (vinho base mais o licor de tiragem). Atualmente, existem diversos trabalho de seleção leveduras específicas e estudos comparativos de cepas para a elaboração desse tipo de produto (Borrull et al., 2015; Di Gianvito et al., 2018; Garofalo et al., 2018, 2016; Gonzalez et al., 2003; Martínez-García et al., 2017; A. Martínez-Rodríguez et al., 2001; Martínez-Rodríguez et al., 2002; Nadal et al., 1999; J. A. Porras-Agüera et al., 2019; Velázquez et al., 2016), além de diversas cepas comerciais comercializadas na forma liofilizada.

A Figura 5 mostra um desenho de uma célula de levedura *S. cerevisiae* no momento da sua replicação mitótica (assexuada), sendo possível visualizar com detalhe, as organelas, compartimentos celulares e formação do broto.

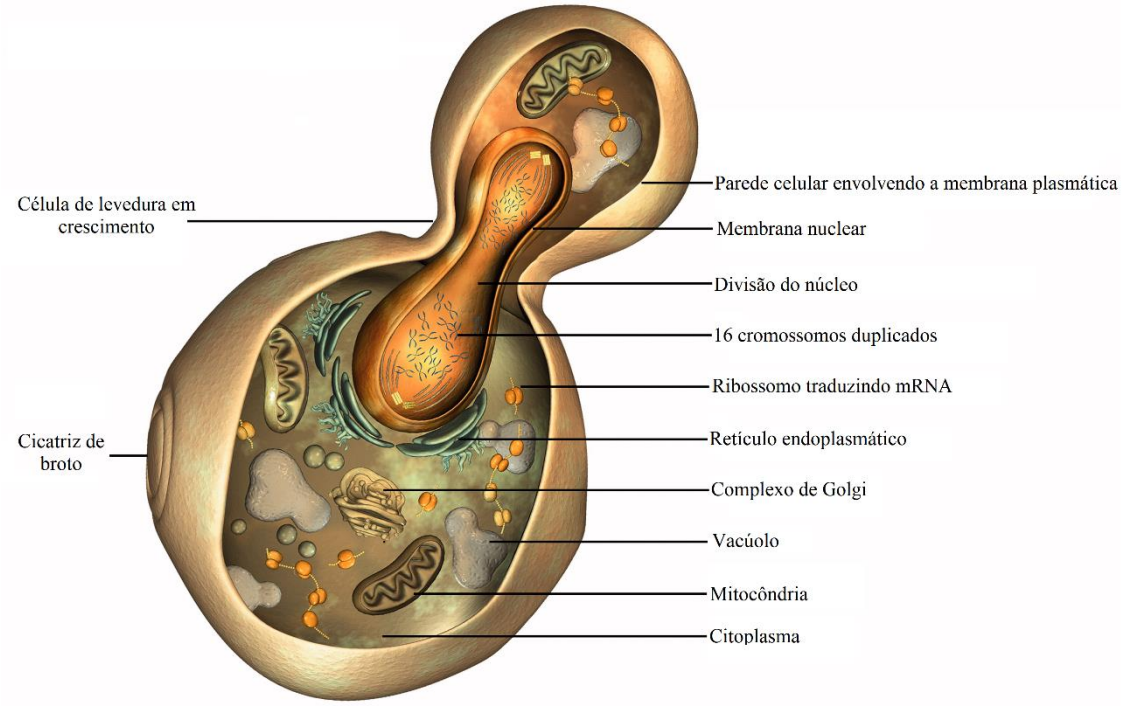

Figura 5: Principais características de uma célula de *Saccharomyces cerevisiae*. Estrutura de uma célula de levedura *Saccharomyces cerevisiae* haploide em reprodução por brotamento (gemiparidade). Adaptado de: (Pretorius and Pretorius, 2017).

Dentre as diversas cepas de leveduras isoladas e comercializadas da espécie *Saccharomyces cerevisiae*, a cepa LALVIN® EC1118 (Lallemand, Canadá) é convencionalmente utilizada na segunda fermentação de espumantes. Conhecida como "*Prise de mousse*" (tomada de espuma), foi isolada na região de *Champagne* (França) e foi depositada na Coleção *Nationale de Cultures de Microorganismes* (Institut Pasteur, França) como cepa I-4215. Foi a primeira levedura vínica a ter seu genoma sequenciado (Novo et al., 2009), fato que facilita a obtenção de resultados moleculares. A cepa EC1118 contém alta atividade de invertase, convertendo rapidamente sacarose a glicose e frutose (Meneses et al., 2002), além de possuir uma alta capacidade autolítica, maior atividade autofágica e uma vida útil cronológica significativamente menor que outras cepas de leveduras (Orozco et al., 2012a; Torresi et al., 2011). Portanto, possuindo características pertinentes para elaboração de espumantes, onde os compostos cedidos pelas leveduras ao longo do tempo de maturação podem influenciar nas características organolépticas do produto (Leroy et al., 1990).

2.3 Fatores de estresse para as leveduras, sobrevivência e morte celular na segunda fermentação de espumantes

2.3.1 Estresse causado pela composição do vinho base de espumante

No início da segunda fermentação de espumantes elaborados pelos métodos Charmat e Tradicional, algumas condições ambientais proporcionadas pelo vinho base são fatores de estresse para as leveduras. Como já comentado, a composição química do vinho base torna o meio inóspito pois o vinho base possui alta concentração de etanol, presença de glicerol, pH baixo (Borrull et al., 2015), presença de dióxido de enxofre (SO_2) (Sudraud et al., 1985), presença de ácido acético (Giannattasio et al., 2013), aumento gradativo de pressão (Porras-Agüera et al., 2020), entre outros fatores (Aranda et al., 2019; Bauer and Pretorius, 2000). Além disso, a tomada de espuma é normalmente conduzida em temperaturas relativamente baixas (12-16 °C) e o meio possui relativamente baixas concentrações de nutrientes (Kemp et al., 2020; Martí-Raga et al., 2015).

O vinho é uma solução hidroalcóolica na qual, após a água, o etanol é o segundo composto mais abundante. Isso causa um efeito inibitório para vários microrganismos. Nas condições da segunda fermentação de espumantes, o etanol é descrito como o principal fator ambiental a ter influência nas respostas transcricionais da levedura (Penacho et al., 2012). Entre vários fatores testados em estudos, o etanol no vinho base foi considerado o principal fator de estresse para leveduras na segunda fermentação de espumantes (Borrull et al., 2015). A tolerância ao etanol varia muito entre as espécies de leveduras (Lin et al., 2020), e dentro da espécie de *S. cerevisiae*, a tolerância varia dependendo da cepa (Borrull et al., 2015). Além disso, presença de etanol pode causar alterações estruturais nas células de leveduras, o que pode impactar a fluidez da membrana plasmática (Navarro-Tapia et al., 2018), e consequentemente na morfologia celular (Dinh et al., 2008).

Outro composto importante apontado como sendo um fator de estresse nos vinhos base de espumantes é o SO_2, usado como conservante desses vinhos até que seja realizado a segunda fermentação alcoólica. O dióxido de enxofre é utilizado como antioxidante e como inibidor microbiano (Blouin and Peynaud, 2006; Gould and Russell, 2003). Sua ação antimicrobiana está intimamente correlacionada com o pH dos vinhos, onde quanto menor for o pH, maior é a fração livre e molecular desse composto, e consequentemente maior é sua eficácia antimicrobiana. A presença de etanol no meio e a força iônica do vinho também podem influenciar na atividade antimicrobiana do SO_2. Uma maior quantidade de etanol e uma menor força iônica aumentam o pK_{a1} (primeira constante de dissociação) do SO_2 e consequentemente

a fração molecular livre (Waterhouse et al., 2016). Uma vez que o dióxido de enxofre é adicionado ao vinho ou a qualquer solução aquosa, em qualquer uma das formas comumente usadas, ele se dissocia em três espécies moleculares: SO_2 molecular ($SO_2 \cdot H_2O$), bissulfito (HSO_3^-) e sulfito (SO_3^{-2}) (Divol et al., 2012). A Figura 6-A mostra a faixa de pH em que se encontram a maioria dos vinhos, juntamente com os tipos de SO_2 em solução aquosa (dependentes do pH) e a Figura 6- B mostra as porcentagens teóricas das frações de SO_2 molecular dependentes das quantidades de SO_2 livre e do pH.

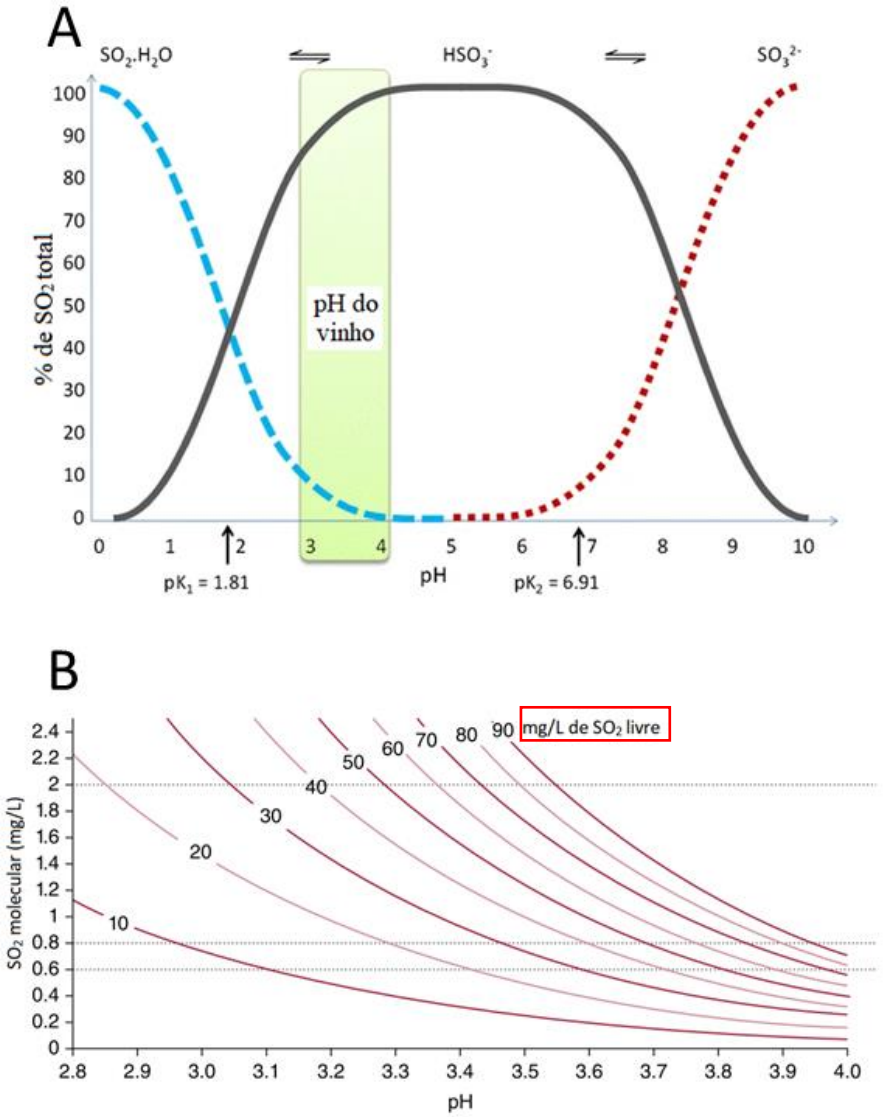

Figura 6: Dissociação do SO_2 e sua interação com pH. A = Formas de SO_2 em solução aquosa dependentes do pH. Adaptado de: (Divol et al., 2012); B= Curvas de concentração para o SO_2 molecular em função do pH para diferentes concentrações de SO_2 livre. As curvas foram determinadas em água pura (pK_a =1,8) utilizando a equação de *Henderson–Hasselbalch* [molecular SO_2 = free SO_2/1 + 10(pH-pK_{a1})]. As linhas pontilhadas entre 0,6 e 0,8 mg/L de SO_2 molecular indicam o intervalo onde normalmente já existe uma ação antimicrobiana do SO_2 molecular (valores próximos para manter os vinhos estocados). Já a linha em 2 mg/L de SO_2 molecular indica o limiar de percepção sensorial. Adaptado de: (Waterhouse et al., 2016).

Segundo a Legislação Brasileira (Lei nº 10970 de 12/11/2004) a concentração máxima permitida é de 350 mg/L de dióxido de enxofre total. No entanto, a legislação não faz nenhuma referência à quantidade de SO_2 livre permitido nos vinhos, ficando a critério do técnico responsável a escolha da quantidade de SO_2 livre para elaboração dos vinhos. Para vinhos tranquilos, tanto para o período de afinamento, como para o posterior engarrafamento uma faixa entre 20 a 30 mg/|L de SO_2 livre se adapta a um grande número de situações (Blouin and Peynaud, 2006). Entretanto, como regra geral, sabe-se que nos vinhos base que serão utilizados para uma segunda fermentação alcoólica, as concentrações ideais de SO_2 livre são inferiores a 10 mg/L (Alexandre, 2019). Contudo, para reduzir os fenômenos oxidativos, contaminações microbianas e fermentações espontâneas, como a fermentação malolática por exemplo que ocasiona uma perda da acidez (transformação do ácido málico em ácido lático por bactérias láticas), nem sempre isto é possível.

Embora vários estudos tenham mostrado alternativas para o SO_2 e redução do seu uso (Capece et al., 2020; Christofi et al., 2021; Hwang et al., 2020; Marchante et al., 2019; Simonin et al., 2020; Zara and Nardi, 2021), até o momento, nenhuma outra técnica física ou aditivo químico pode fornecer a eficácia e amplo espectro de ação deste composto (Lisanti et al., 2019). Um fato interessante é que as leveduras enológicas, particularmente da espécie *S. cerevisiae*, possuem certa tolerância ao SO_2 (García-Ríos and Guillamón, 2019) e essa tolerância varia entre cepas (Nadai et al., 2016). Essa tolerância é um traço desejado nas leveduras de vinho e foi inconscientemente selecionada em práticas vinícolas ao longo do tempo (Zimmer et al., 2014), podendo ser considerada uma vantagem evolutiva (García-Ríos et al., 2019).

A inibição microbiana pelo SO_2 em leveduras tem sido atribuída a diversas alterações celulares, como a modificação da atividade de transporte de membrana por ligação com as proteínas de membrana (Divol et al., 2012), a inibição do glicealdeído-3-fosfato desidrogenase (GAPDH) - uma enzima crítica na via da glicólise (Hinze and Holzer, 1986) e outras enzimas como ATPase e álcool desidrogenase (ADH) (Maier et al., 1986), causando uma diminuição do conteúdo ATP nas células (Hinze and Holzer, 1986; Maier et al., 1986; Schimz and Holzer, 1979). Além disso, ocorre a modificação da expressão de muitos genes correlacionados com o metabolismo celular (Park and Hwang, 2008), degradação da tiamina disponível (Labuschagne and Divol, 2021), e ligação de metabólitos (acetaldeído, piruvato, glicose, dihidroxilato-fosfato, ácido oxalocético e ácido α-cetoglutárico), impedindo assim seu uso adicional como substratos para vias metabólicas (Rankine and Pocock, 1969).

A Figura 7 ilustra um esquema com alguns mecanismos utilizados pela levedura para diminuir o estresse causado pelo SO_2, além da via metabólica do enxofre como precursor de alguns aminoácidos essenciais. A figura mostra ainda diversos mecanismos de entrada e saída dessas moléculas e o SO_2 molecular entrando na célula de maneira facilitada.

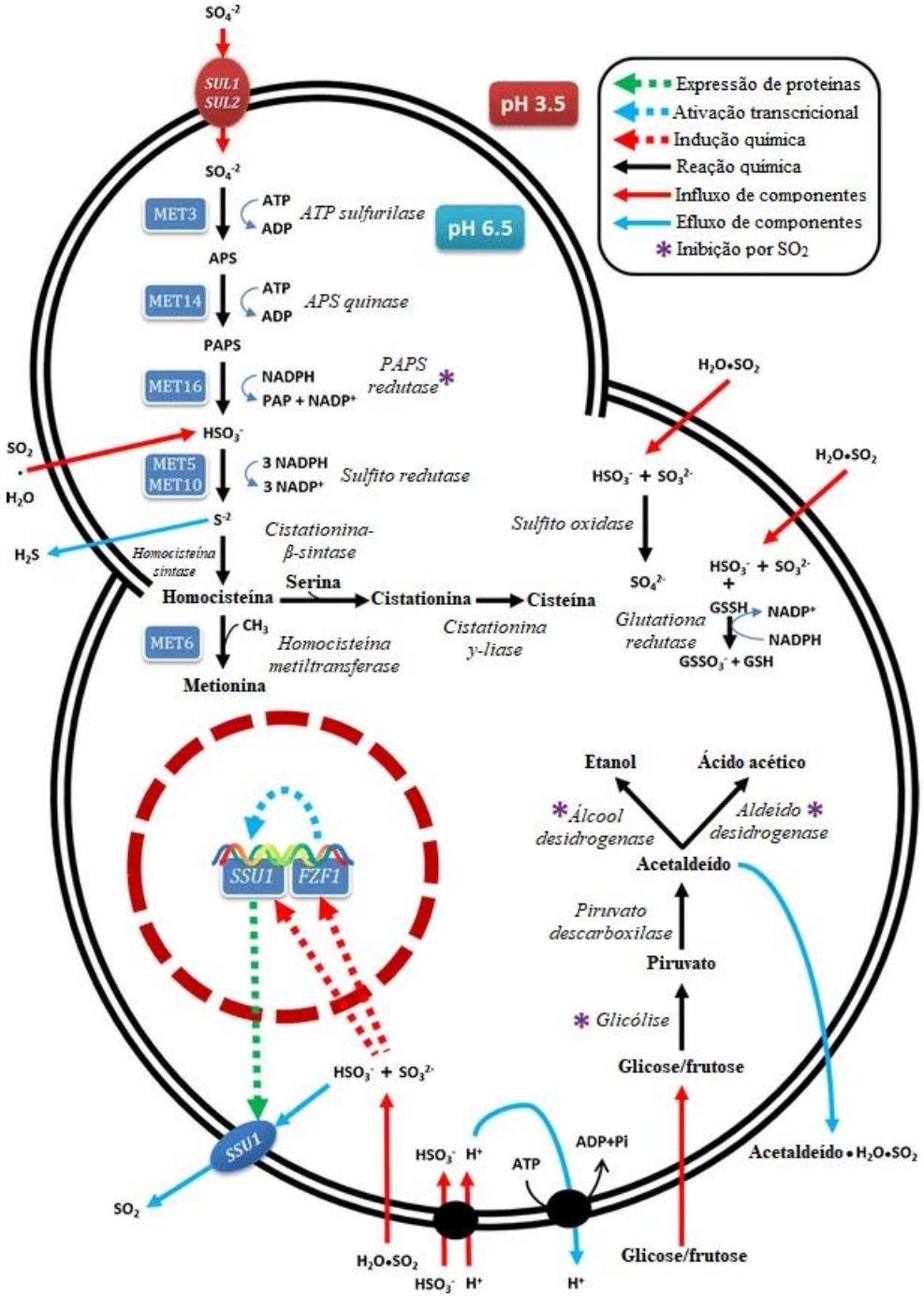

Figura 7: Respostas celulares e moleculares de *S. cerevisiae* na presença de SO_2. Adaptado de:(Divol et al., 2012).

Existem diversos genes envolvidos no estresse causado pelos sulfitos (Figura 7). O gene *SSU1* é um gene relacionado à resistência a sulfito, que codifica uma proteína chamada Ssu1p.

A proteína Ssu1p é uma bomba de efluxo de sulfito, o que significa que ela atua como um transportador para remover o sulfito do interior das células. Essa proteína está localizada na membrana plasmática das células de *Saccharomyces cerevisiae*. Quando as leveduras estão expostas a altos níveis de sulfito, o gene *SSU1* é ativado e começa a produzir a proteína Ssu1p em maior quantidade. A presença dessa proteína ajuda a proteger as células contra os efeitos tóxicos do sulfito, permitindo que as leveduras sobrevivam em ambientes com altos níveis desse composto (Divol et al., 2012). O gene *FZF1* é um gene que codifica uma proteína chamada Fzf1p, podendo ser ser um ativador transcricional para o gene *SSU1* (Avram et al., 1999; Divol et al., 2012). Além disso, a proteína Fzf1p está envolvida na resposta ao estresse oxidativo e desempenha um papel fundamental na regulação da expressão de genes relacionados à resposta a essa condição. O estresse oxidativo ocorre quando as células estão expostas a altos níveis de espécies reativas de oxigênio, que podem danificar componentes celulares, como lipídios, proteínas e DNA (Sarver and DeRisi, 2005). Os genes *SUL1* e *SUL2* codificam proteínas transportadoras de compostos enxofrados, conhecidas como Sul1p e Sul2p, respectivamente. Essas proteínas estão localizadas na membrana plasmática das células de leveduras *Saccharomyces cerevisiae*. As proteínas Sul1p e Sul2p são responsáveis pelo transporte ativo de sulfato para o interior das células. O sulfato é uma forma oxidada de enxofre amplamente encontrada no ambiente. Nas células de *Saccharomyces cerevisiae*, o sulfato é uma importante fonte de enxofre para a síntese de aminoácidos sulfurados, como a cisteína e a metionina (Chen et al., 2018). Na via do metabolismo do enxofre, como é possível visualizar na Figura 7, há participação de uma série de genes e enzimas. O gene *MET14*, por exemplo, desempenha um papel intermediário crucial na via metabólica de biossíntese dos aminoácidos metionina e cisteína nas células de *Saccharomyces cerevisiae*. Ele codifica a proteína Met14p, que está diretamente envolvida na síntese desses aminoácidos. A proteína Met14p exerce uma função essencial na regulação e no metabolismo desses aminoácidos dentro da célula. (Hansen and Francke Johannesen, 2000).

2.3.2 Autofagia em leveduras

Autofagia diz respeito ao conjunto de processos que envolvem a degradação de componentes citoplasmáticos, incluindo o citosol, complexos macromoleculares, e organelas, dentro de vacúolos ou lisossomos. A autofagia está relacionada aos processos celulares básicos de rearranjo de estruturas subcelulares e componentes necessários à sobrevivência e homeostase celular (reutilização de proteínas, ácidos nucléicos etc.). Sendo assim, a autofagia é um

fenômeno “pró-vida”, mas em determinadas condições, pode levar à morte celular. Vários tipos de autofagia ocorrem em células eucarióticas. Os processos e sistemas regulatórios básicos relacionados com a autofagia são altamente conservados, de tal forma que a compreensão da cascata de eventos envolvidos na autofagia em leveduras ajuda a desvendar fenômenos em outros organismos, incluído o homem (Reggiori and Klionsky, 2013).

A autofagia pode ser dividida em dois grupos: a microautofagia e a macroautofagia. A microautofagia está envolvida na reutilização de mitocôndrias, partes do núcleo, e peroxissomos. Neste caso, os componentes são recrutados e sequestrados diretamente pela membrana vacuolar e posteriormente liberados no lume do vacúolo. Já na macroautofagia, os componentes a serem reciclados são primariamente fechados em vesículas de membrana dupla (autofagossomo) que por sua vez se fundem com o vacúolo possibilitando o reciclo de macromoléculas. As vias autofágicas Cvt (Citoplasma para vacúolo alvo) envolvem um amplo número de genes altamente regulados, associados à formação de vesículas, acoplamento de componentes, enzimas hidrolíticas intra-vacuolres e outros (Reggiori and Klionsky, 2013).

Na enologia a autofagia possui um importante papel na liberação de compostos da levedura para os vinhos (Cebollero et al., 2005). Autofagia é um caminho de degradação ativado pela falta de nitrogênio ou carbono (Huang and Klionsky, 2002). Em um estudo (Cebollero et al., 2005), os pesquisadores utilizaram um mutante de levedura com um gene defeituoso relacionado com o processo autofágico, com o intuito de mostrar que a autofagia ocorre nas condições de produção de vinhos. Os autores concluíram que a autofagia ocorre nessas situações específicas onde o vinho é submetido a um afinamento sobre as borras. Portanto, genes relacionados à autofagia são bons candidatos para se estudar a base molecular da autólise ou para a engenharia genética de leveduras enológicas.

A Figura 8 mostra uma visão geral dos estágios da autofagia com as principais proteínas envolvidas no processo de formação de membranas autofagossômicas.

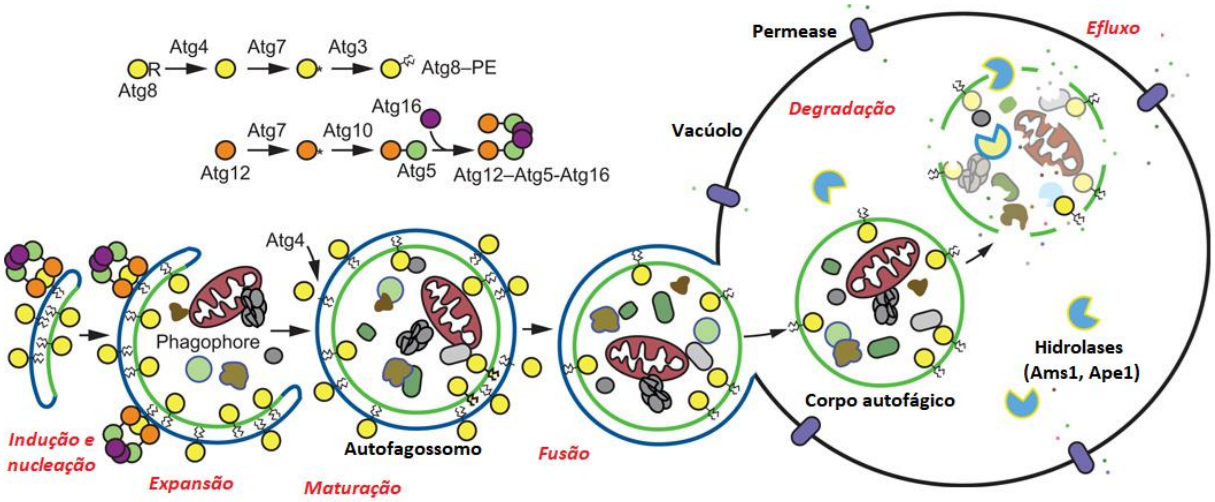

Figura 8: Uma visão geral da via autofágica em *S. cerevisiae*. A figura mostra uma visão esquemática de cada um dos quatro estágios principais da autofagia: indução e nucleação do fagóforo, expansão do fagóforo e fechamento para formar o autofagossomo, fusão autofagossomo com o vacúolo e degradação e efluxo dos compostos. Antes do alongamento da membrana do fagóforo, o Atg8 é convertido em sua forma lipídica, Atg8–PE após uma série de eventos proteolíticos envolvendo o sistema de conjugação Atg8 (Atg3, Atg4, Atg7 e Atg8). O sistema de conjugação tipo ubiquitina Atg12 (Atg5, Atg7, Atg10, Atg12 e Atg16) também é necessário para o alongamento da membrana. Adaptado de: (Delorme-Axford et al., 2015).

A Figura 9 mostra uma representação dos dois principais tipos de autofagia, microautofagia e macroautofagia.

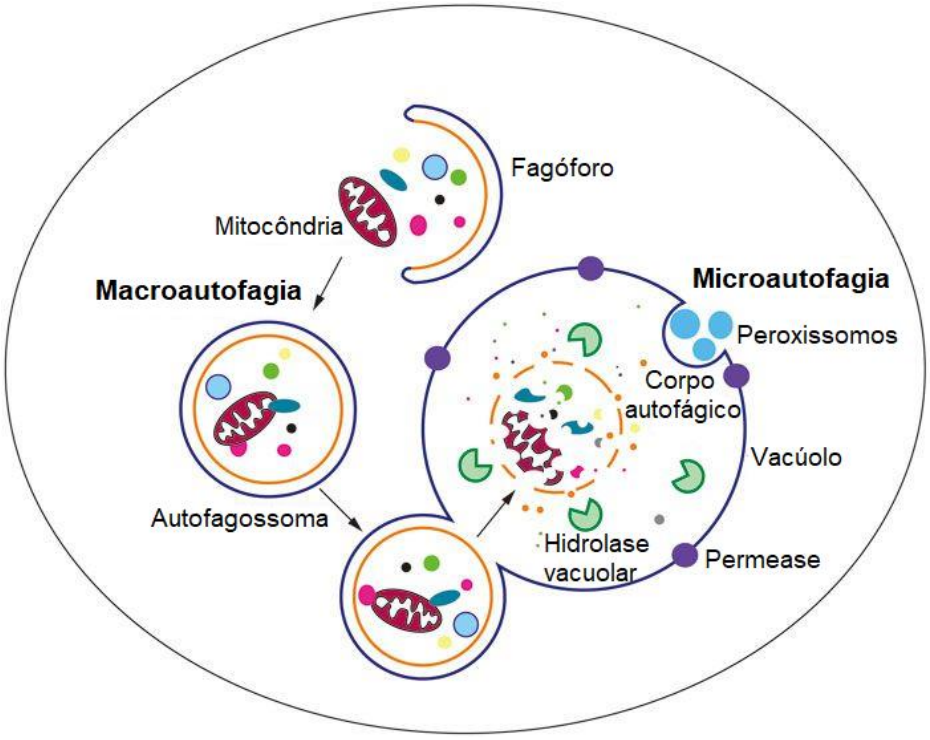

Figura 9: Representação dos dois principais tipos de autofagia em leveduras. Na macroautofagia, as organelas do citoplasma são sequestradas pelo fagóforo em expansão, levando à formação do autofagossomo. Após a conclusão,

o autofagossomo se funde com o vacúolo, liberando a vesícula interna do autofagossomo no lúmen do vacúolo, onde é agora denominado corpo autofágico. Durante a microautofagia (aqui ilustrada a micropexofagia como exemplo), as estruturas direcionadas para a degradação são agrupadas na proximidade da membrana do vacúolo, sendo absorvidas por invaginação da membrana do vacúolo, ficando expostas internamente às enzimas hidrolases vacuolares, ocorrendo subsequente cisão, lise e degradação. Os metabólitos resultantes, são aminoácidos, açúcares e nucleotídeos, que são subsequentemente transportados para o citoplasma por permeases e usados como fonte de energia ou como blocos de construção para a síntese de novas macromoléculas. Adaptado de: (Feng et al., 2014).

2.3.3 Apoptose em leveduras

O termo apoptose significa morte celular programada. Essa morte programada é um processo essencial para a manutenção do desenvolvimento dos seres vivos multicelulares, pois eliminas as células que por alguma razão não estão funcionando corretamente (Meier et al., 2000), entretanto pode ocorrer também em organismos unicelulares como as leveduras por exemplo (Fröhlich and Madeo, 2000; Madeo et al., 1997). A primeira vez em que o fenômeno apoptótico foi descrito em um organismo unicelular (*Saccharomyces cerevisiae)* ocorreu no ano de 1997 (Madeo et al., 1997). Os genes que funcionam na morte celular das leveduras foram confirmados como reguladores apoptóticos de seres pluricelulares, indicando que o sistema básico que desencadeia a apoptose está também presente nas células de *S. cerevisie.* A partir desse trabalho, as leveduras tem sido usadas como um sistema modelo de apoptose por vários grupos de pesquisa em diversas áreas do conhecimento (Büttner et al., 2006; Carmona-Gutierrez et al., 2010; Fabrizio et al., 2004; Jin and Reed, 2002; Lone et al., 2020; Madeo et al., 2004, 2002; Rego et al., 2013; Scariot et al., 2016; Zhang et al., 2007).

Em ambos os casos (seres unicelulares ou pluricelulares) a célula morre em razão de um conjunto de mudanças coordenadas, caracterizando um processo ativo. A apoptose pode ter um papel importante também como um mecanismo de defesa (remoção de células parasitadas ou malignas por apoptose, controle da homeostase entre outros) (Fröhlich and Madeo, 2000; Junqueira and Silva Filho, 2012). Entre os organismos unicelulares, a morte programada das células, serve como um mecanismo de preservação das populações de células em face a um estresse (insuficiência de nutrientes e outras condições adversas), garantindo que parte das células sobrevivam para propagar seu genoma (Fröhlich and Madeo, 2000).

Segundo Madeo, et al. (1999), a formação de ERO (Espécie Reativas de Oxigênio) é um evento-chave no mecanismo apoptótico evolutivo dos seres vivos. Em *S. cerevisiae*, exposição a baixas doses de H_2O_2 ou acúmulo de ERO por esgotamento da glutationa também induz a apoptose (Madeo et al., 1999). Essas espécies reativas de oxigênio são subprodutos da respiração e ocorrem em todos os organismos aeróbicos. A ocorrência desse fenômeno é

frequente e modifica proteínas, lipídios e ácidos nucleicos, portanto, a ocorrência de apoptose em organismos unicelulares, poupa recursos de nutrientes para as células circundantes mais adaptadas, jovens e saudáveis, sendo assim, vantajoso para seus parentes clonais (Fröhlich and Madeo, 2000; Madeo et al., 1999).

2.3.4 Necrose em leveduras

Diferente da morte por apoptose que evita a ruptura da célula e o derramamento de conteúdo celular, a morte celular por necrose é geralmente acompanhada de quebra celular, inflamação e ruptura da membrana plasmática, seguido pela perda de conteúdo intracelular (Fröhlich and Madeo, 2000). O fenômeno de morte celular por necrose incialmente tinha sido definido como um tipo de morte que não tem as características de apoptose e autofagia, e eram geralmente consideradas descontroladas e induzidas por lesões externas, como exemplo, o contato com moléculas tóxicas (fungicidas) (Scariot et al., 2018, 2017). Entretanto, pesquisas mais recentes sugerem que existem etapas de ocorrência que podem ser rigorosamente reguladas e que são consideradas como morte por necrose programada (Conrad et al., 2016; Eisenberg et al., 2010; Golstein and Kroemer, 2007; Zong and Thompson, 2006), tanto em mamíferos como em células de leveduras (Carmona-Gutiérrez et al., 2011; Falcone and Mazzoni, 2016; Galluzzi et al., 2012). Nesse caso, a necrose programada pode ser considerada como um mecanismo de comunicação e poderia servir como uma espécie de sinalizador através da liberação dos compostos intracelulares (Eisenberg et al., 2010).

2.3.5 Autólise das leveduras

O envelhecimento das células e posterior morte, são fenômenos relevantes na produção de espumantes. Nesse ambiente a lise celular pode contribuir para as propriedades organolépticas finais do vinho (Ribéreau-Gayon et al., 2021). A autólise da levedura pode ser definida como a hidrólise de biopolímeros sob a ação de enzimas hidrolíticas que liberam o conteúdo citoplasmático (peptídeos, aminoácidos, ácidos graxos e nucleotídeos) e compostos da parede celular (glucanos, manoproteínas). Geralmente, a autólise natural ocorre no final da fase estacionária de crescimento e está associada com a morte celular. (Alexandre and Guilloux-Benatier, 2006; Babayan and Bezrukov, 1985; Moreno-Arribas and Polo, 2009). As enzimas hidrolíticas do tipo proteases estão entre as principais responsáveis pelo processo de autólise, podendo ser responsáveis por mais de 60-80 % do nitrogênio liberado durante o processo. (Alexandre, 2011).

Durante o contato do vinho com as leveduras no processo de maturação dos espumantes ocorre um enriquecimento de componentes derivados da autólise. Muitos fatores influenciam a qualidade e quantidade de compostos liberados no vinho durante o curso da autólise, sendo que os mais importantes incluem a cepa de levedura utilizada, suas condições de crescimento e população, temperatura de armazenamento, teor de etanol, pH do vinho e duração do contato com leveduras (Babayan and Bezrukov, 1985; Leroy et al., 1990). Esses fatores também influenciam a atividade enzimática das proteases (Alexandre, 2011). Os principais constituintes formados e liberados durante a autólise incluem compostos nitrogenados, polissacarídeos, componentes de ácido nucleico, ácidos graxos, várias vitaminas e compostos aromáticos. Estes e outros componentes das leveduras jogam um papel importante na qualidade do vinho espumante, modificando as propriedades organolépticas e a qualidade da espuma do produto final (Alexandre, 2011; Alexandre and Guilloux-Benatier, 2006).

As mudanças bioquímicas que ocorrem durante o período em que o vinho fica em contato com as leveduras são as seguintes: inicialmente, após a segunda fermentação, ocorre uma liberação passiva de aminoácidos. Após 3 a 6 meses, o enriquecimento do meio em aminoácidos continua devido à hidrólise de peptídeos e proteínas junto de um aumento significativo dos polissacarídeos da parede celular, iniciando a degradação da membrana plasmática e a liberação de lipídios no meio. Após 9 a 12 meses, a liberação de aminoácidos diminui, aumentando a liberação de proteínas e peptídeo, além do aumento dos polissacáridos de parede celular, lipídios e ribonucleotídeos (Alexandre, 2011). Esses detalhes podem ser vistos esquematicamente na Figura 10.

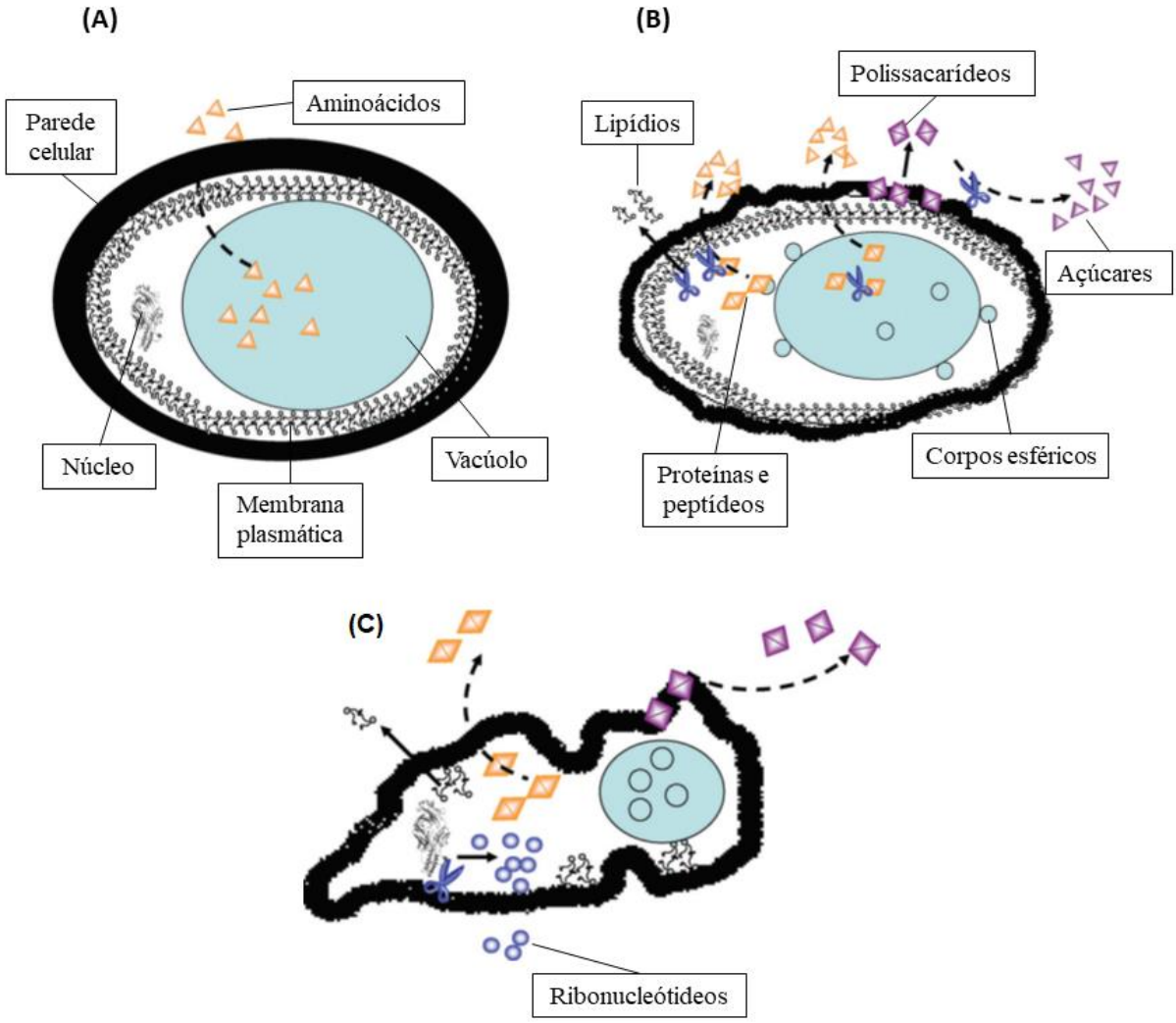

Figura 10: Representação esquemática das mudanças morfológicas e bioquímicas que ocorrem durante a autólise em espumantes. (**A**) após a segunda fermentação alcoólica, as células de levedura são alongadas e ovoides com a parede celular grossa e lisa. Dentro da célula, um grande vacúolo cercado por corpos esféricos. (**B**) entre 3 e 6 meses, a célula e o vacúolo diminuem. Corpos esféricos são distribuídos por todo o vacúolo. A parede celular é áspera, e pequenas rugas ou dobras são observadas. (**C**) entre os 9 a 12 meses, a célula aparece murcha, o que explica o menor tamanho. A parede celular permanece integra, porém com muitos cumes e dobras perdendo grande parte de seu conteúdo citoplasmático. Fonte: Adaptado de (Alexandre, 2019, 2011).

Esse mecanismo de autólise de levedura mostrado na Figura 10 é válido para os processos de autólise natural em vinhos espumantes, onde o pH é em torno de 3 a 4 e a temperatura de envelhecimento próximo de 15 ºC, longe das condições ideais para essas enzimas (pH=5 e temperatura próxima de 45 ºC) (Alexandre, 2011). O processo de autólise induzida existe e é muito explorado pela indústria na fabricação extratos de levedura, produção de enzimas e outros. A autólise induzida pode ser realizada por exemplo por meio de indutores físicos como o aumento e diminuição de temperatura alternados, aumento de pressão (Babayan

and Bezrukov, 1985; Comuzzo et al., 2017), indução por campo elétrico pulsado (Martínez et al., 2016) entre outros (Gnoinski et al., 2021b). Já os indutores químicos podem ser por exemplo, mudanças de pH, utilização de enzimas exógenas, utilização de detergentes e antifúngicos. Dependendo do indutor utilizado, o processo de autólise pode ser rápido, durando entre 48 a 72 horas (Alexandre, 2011; Gnoinski et al., 2021b).

A Figura 11 mostra células de leveduras *S. cerevisiae* em diferentes etapas do processo fermentativo e pós-fermentativo. É possível observar as mudanças fisiológicas das células, caracterizado pelo esvaziamento intracelular, tornando a célula "murcha".

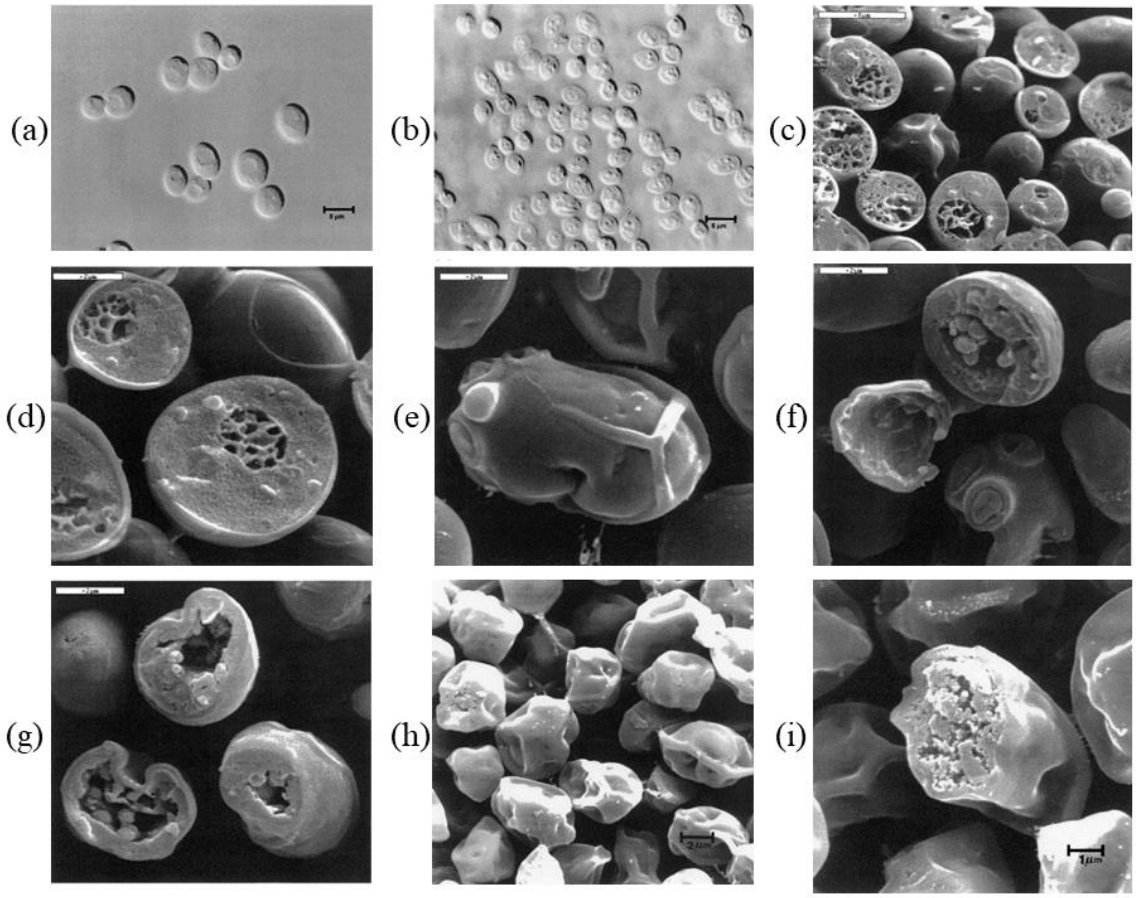

Figura 11: Imagens de microscopia relacionadas com a autólise de leveduras. (**a**), (**b**) - Microscopia de Luz com óptica de Contraste de Interferência Diferencial (DIC) ou óptica Nomarski; (**c**), (**d**), (**e**), (**f**), (**g**), (**h**), (**i**) - Microscopia Eletrônica de Varredura de Baixa Temperatura (LTSEM); de células de leveduras *Saccharomyces cerevisiae* linhagem LALVIN® EC1118 (Lallemand, Canadá). (**a**) Células de levedura 20 dias após o início da segunda fermentação de espumante; (**b**) Células de levedura 12 meses de contato com o espumante, após a fermentação; (**c**), (**d**) imagens de leveduras cultivadas em meio sintético durante 24 horas parcialmente fraturadas, sendo possível visualizar a estrutura granular do citoplasma; (**e**), (**f**), (**g**) células após 24 horas de autólise induzida em um sistema de vinho modelo. Nas células fraturadas é possível visualizar uma perda do conteúdo citoplasmático; (**h**) (**i**) Imagens de leveduras após 12 meses de contato com o espumante após fermentação. Neste

caso se observa uma diminuição da turgidez e na célula fraturada uma degradação dos componentes celulares internos. Adaptado de: (A. J. Martínez-Rodríguez et al., 2001).

2.4 Marcadores de estresse

2.4.1 Avaliação celular

A determinação da viabilidade celular é o método mais utilizado para avaliar o impacto de vários tipos de estressores na pesquisa de toxicidade e em estudos de microbiologia industrial (Kwolek-Mirek and Zadrag-Tecza, 2014). A viabilidade é definida como uma porcentagem de células vivas em toda uma população. A avalição da viabilidade e crescimento celular pode ser realizada de diversas maneiras. Os modos mais convencionais são utilizando microscópios óticos, hemocitômetros e corantes de exclusão, ou crescimento de colônias em placas onde crescem células viáveis e cultiváveis (unidades formadoras de colônias) em um meio de cultura sólido (YPD + ágar) (Fugelsang and Edwards, 2007).

Populações viáveis de leveduras podem ser estimadas contando-as diretamente no microscópio usando técnicas específicas de coloração ou epifluorescência (Ribéreau-Gayon et al., 2021). A medição da viabilidade celular utilizando azul de metileno como corante das células é uma técnica relativamente antiga (Gilliland, 1959). Tradicionalmente usado nas indústrias de vinho e de cerveja, o azul de metileno penetra em células vivas e mortas e existe em dois estados dependentes de redox: reduzido e oxidado. O estado reduzido é incolor, enquanto a forma oxidada fica azul (Stewart, 2017). Portanto, quando a célula de levedura viável absorve o corante, ele é reduzido enzimaticamente à forma incolor, enquanto, nas células mortas, elas não reduzem o corante e a célula permanece azul ou preta. Assim, presume-se que as células que reduzem o corante para a forma incolor sejam viáveis (Fugelsang and Edwards, 2007). Utilizando microscopia de fluorescência ou citômetro de fluxo é possível utilizar o iodeto de propídio que é um corante intercalante nuclear e cromossômico que emite fluorescência na cor vermelha. A emissão da fluorescência dependente de alterações na integridade e funcionalidade da membrana plasmática da célula. Esse corante no pH fisiológico é aniônico e não penetrar nas células vivas devido à carga negativa da membrana celular (Stewart, 2017). Como o iodeto de propídio não permeia a membrana celular integra (células vivas), é comumente utilizado para detectar células mortas ou com membrana danificada em uma população de células (Longin et al., 2017). A Figura 12 mostra diferentes formas de avaliar a viabilidade celular de uma população de leveduras.

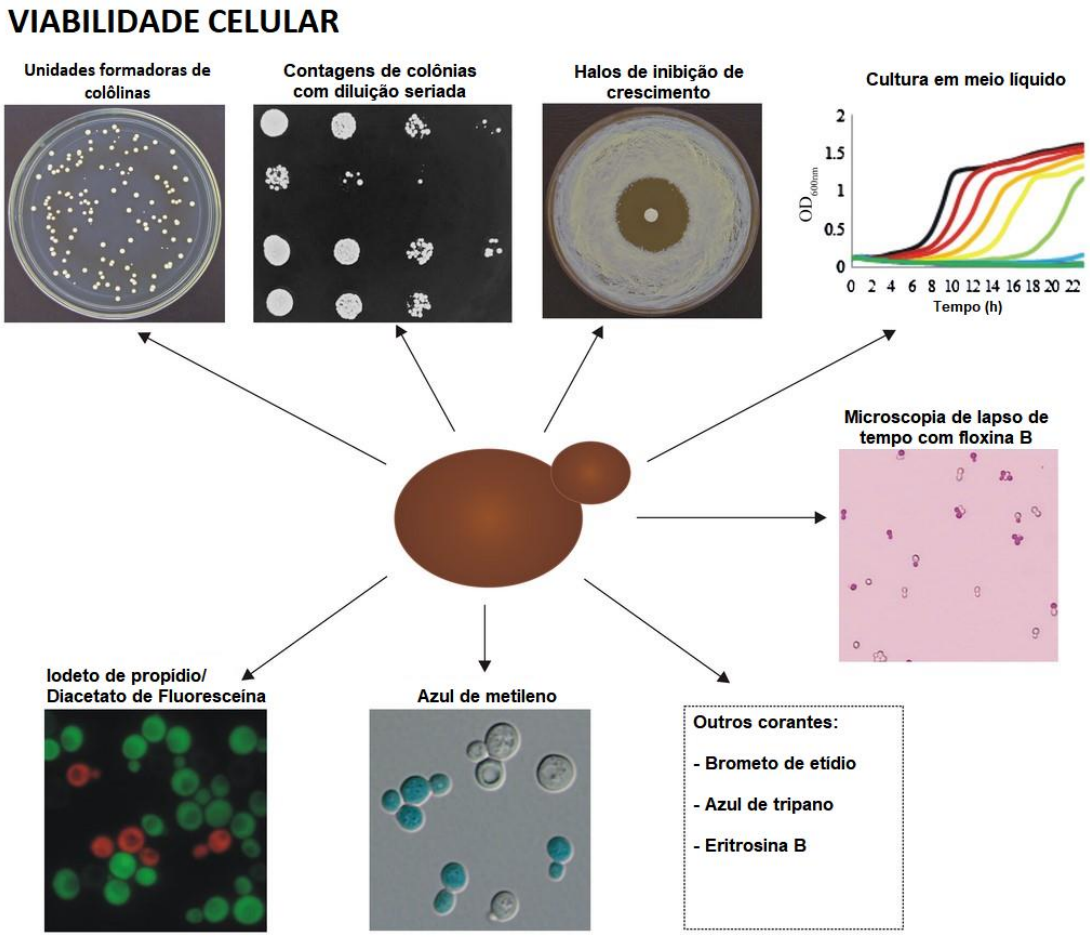

Figura 12: Diferentes formas de se avaliar a viabilidade da população de células de leveduras. Adaptado de (Kwolek-Mirek and Zadrag-Tecza, 2014).

A análise de várias funções celulares diferentes durante a fermentação permite ao operador estabelecer correlações entre atributos celulares e fazer previsões sobre a condição geral e o nível de resposta ao estresse da cultura (Sommer, 2020). Embora a morte celular seja uma das consequências da toxicidade, fatores químicos ou físicos podem exercer seus efeitos tóxicos através de uma série de alterações celulares que podem comprometer a capacidade celular de se dividir sem necessariamente levar à morte celular. Esse aspecto representa o termo "vitalidade celular" definido como capacidades fisiológicas das células. É importante notar que a viabilidade celular e a vitalidade celular representam dois aspectos diferentes das funções celulares, e ambos são necessários para a estimativa do estado fisiológico de uma célula após a exposição a vários tipos de estressores e fatores químicos ou físicos (Kwolek-Mirek and Zadrag-Tecza, 2014). Uma das formas de avaliar a vitalidade celular é medir a atividade metabólica com o composto fluorescente 5-diacetato carboxifluoresceína (CFDA) que mede a atividade enzimática (esterases) que é necessária para ativar sua fluorescência (Boone et al., 2017; Dorsey et al., 1989).

A citometria do fluxo é uma tecnologia baseada em laser e é usada principalmente para medir a intensidade da fluorescência ou a luz dispersa, obtendo informações das células, possibilitando analisar suas propriedades físicas (Halder et al., 2018). É uma técnica normalmente usada para análise de células de mamíferos, porém atualmente a citometria de fluxo está sendo aplicada em células bacterianas e fúngicas, bem como espécies biomoleculares relevantes para a segurança alimentar (Taitt and North, 2014), e também para avaliar e quantificar microrganismos no vinho (Longin et al., 2017; Sizzano and Blackford, 2022; Sommer, 2020). A citometria de fluxo não apenas monitora o desempenho da fermentação de uma porção estatisticamente relevante da população de leveduras, mas também identifica possíveis problemas de fermentação no nível celular antes que eles afetem toda a cultura, tornando essa tecnologia uma ferramenta de controle de qualidade verdadeiramente flexível (Sommer, 2020). Portanto, para um controle fisiológico e compreensão dos estresses causados nas células, além da viabilidade e vitalidade celular, análises como pH intracelular, potencial de membrana mitocondrial, espécies reativas de oxigênio, entre outras, podem ser avaliadas com o citômetro de fluxo de forma rápida e eficiente (Longin et al., 2017).

A citometria de fluxo tem um enorme potencial como ferramenta de controle de processos nas vinícolas e pode melhorar a compreensão do comportamento da levedura durante a fermentação (Sizzano and Blackford, 2022). Esta é uma técnica que analisa as células isoladamente em um fluxo de líquido, sendo um método individual, quantitativo e qualitativo utilizado para a caracterizar partículas em suspensão. Trata-se de uma técnica usada para analisar múltiplos parâmetros de células individuais dentro de populações heterogêneas. Resumidamente um citômetro do fluxo é composto por três componentes principais: sistema fluídico, ótico, e eletrônico, como mostra a Figura 13 (Longin et al., 2017).

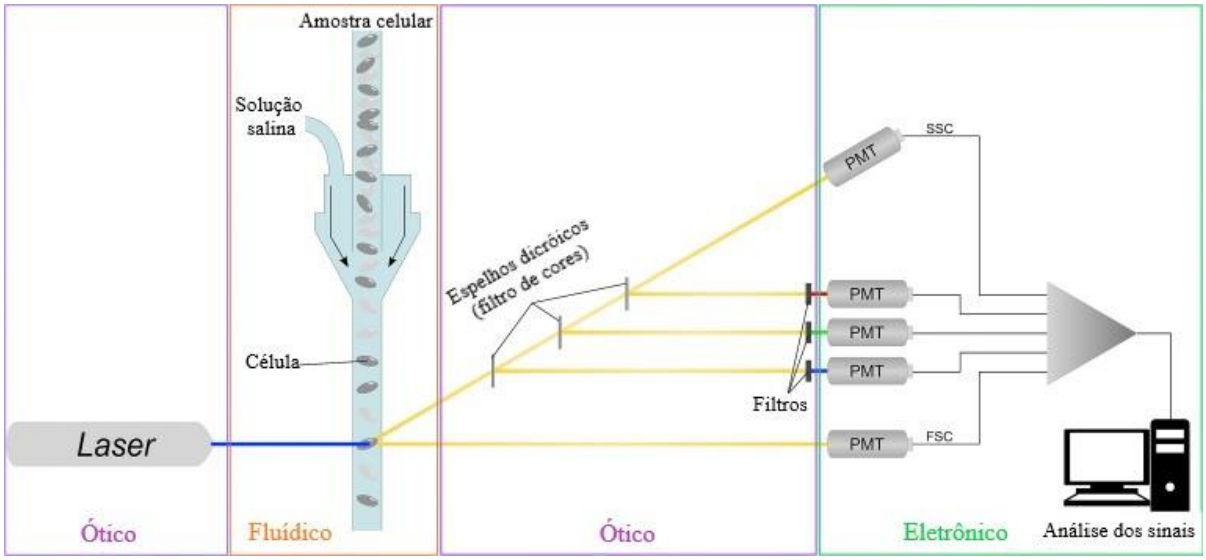

Figura 13: Citômetro de fluxo composto por sistemas fluídico, óptico e eletrônico. PMT = *photomultiplier tubes* (tubos fotomultiplicadores); Posições dos feixes de luz: SSC= *Side-scattered* (dispersão lateral da luz) e FSC= Forward scatter (dispersão frontal da luz). Adaptado de (Longin et al., 2017).

2.4.2 pH intracelular e espécies reativas de oxigênio (ERO)

O pH intracelular tem um papel importante na manutenção das funções normais das células de levedura. A capacidade da célula em manter a homeostase do pH em resposta às mudanças ambientais tem ganhado cada vez mais interesse em pesquisas básicas e aplicadas (Bassnett et al., 1990; Imai and Ohno, 1995; Valli et al., 2005; Weigert et al., 2009). A homeostase do pH intracelular parece estar envolvida na preservação do equilíbrio celular em resposta a diversos fatores ambientais e condições de estresse, como: condições nutricionais (Orij et al., 2011), presença de etanol (Rosa and Sá-Correia, 1996), choque hiperosmótico (Vindeløv and Arneborg, 2002), choque térmico (Piper et al., 1997) e presença de ácidos orgânicos fracos (Bassnett et al., 1990) por exemplo. Há um interesse crescente na medição do pH intracelular ou, mais geralmente, na investigação da capacidade da célula de levedura de manter homeostase mesmo em resposta a diferentes condições de estresse (Orij et al., 2011). Diversos estudos foram realizados utilizando diferentes formas de quantificar o pH intracelular das células (Aon and Cortassa, 1997; Carmelo et al., 1997; Karagiannis and Young, 2001; Valli et al., 2005). Atualmente existem corante fluorescentes específicos para medir o pH intracelular em células vivas. O pHrodo Green por exemplo ((Thermo Fisher Scientific, MA, EUA) é um corante fracamente fluorescente em pH neutro e cada vez mais fluorescente à medida que o pH diminui.

As espécies reativas de oxigênio (ERO) são compostos químicos resultantes da ativação ou redução do oxigénio molecular ou derivados dos produtos da redução. São considerados como marcadores de estresse oxidativo (Farrugia and Balzan, 2012), e abrangem uma ampla

gama de moléculas derivadas do oxigênio molecular, como o radical superóxido ($O_2^{\bullet-}$), peróxido de hidrogênio (H_2O_2) e o radical hidroxila ($OH^{\bullet}$). Sendo que o acúmulo de ERO é um contribuinte para a morte celular (Farrugia and Balzan, 2012; Preiss et al., 2018), podendo estar associado a morte celular programada (Perrone et al., 2008).

Estresses nutricionais e ambientais, como o esgotamento do nitrogênio, levaram ao acúmulo de ERO nas células e podem afetar a fermentação (Mendes-Ferreira et al., 2010). Esses autores mostraram uma estreita relação entre o aumento do acúmulo de espécies de oxigênio reativo e os efeitos negativos na integridade da membrana celular. Exposição a metais pesados (Xu et al., 2010), irradiação ultravioleta (UV), herbicidas, poluentes atmosféricos e outros fatores exógenos também podem induzir uma geração significativa de ERO (Gille and Sigler, 1995; Halliwell and Cross, 1994). Em um trabalho sobre o estresse do etanol em leveduras (Jing et al., 2018) os autores mostraram uma maior ativação e produção de ERO (H_2O_2 e $O_2^{\bullet-}$) em leveduras sob o estresse de etanol, correlacionando esse aumento de ERO com o aumento de autofagia nas células, como uma forma de eliminar esses radicais livres. Em um experimento sobre tolerância das leveduras ao estresse do dióxido de enxofre (Lage et al., 2019), os pesquisadores relatam maior acúmulo de ERO no citosol na presença de SO_2, sugerindo que ocorra uma indução ao estresse oxidativo.

Uma forma de medir ERO em uma célula de levedura com citometria de fluxo é utilizando corantes fluorescente como por exemplo o diclorofluoresceína diacetato (DCFH-DA). Esse é um método baseado na utilização do corante não fluorescente 2'-7'-diclorofluoresceína diacetato (DCFH-DA), a qual atravessa facilmente a membrana celular e sofre desacetilação por ação de enzimas esterases intracelulares, e se oxida pelos ERO presentes na célula, formando o derivado oxidado fluorescente 2',7'-diclorofluoresceína (DCF). Trata-se de uma medida indireta para a produção total de ERO dentro de uma célula, uma vez que a molécula só é detectada após entrar na célula, ser desacetilada e ser oxidada por ERO (Pérez-Gallardo et al., 2013; van der Laan et al., 2020).

2.4.3 Expressão gênica

Todas as condições de estresse causam reações nas células, podendo haver mudanças no metabolismo com ativação ou desativação, aumento ou diminuição da expressão de diferentes genes (Moreno et al., 2019; Zyrina et al., 2017), isso causa a síntese de diferentes proteínas dependendo da etapa do processo (Porras-Agüera et al., 2019), podendo ocorrer formação de espécies reativas de oxigênio (Orozco et al., 2012a), início de processos de autofagia (Reggiori and Klionsky, 2013), apoptose (Madeo et al., 1997), necrose (Scariot et al., 2018), entre outros

eventos. Como consequência final do estresse podem ocorrer mutações, mau funcionamento, inanição ou morte celular. Por esse motivo, estudar a expressão de genes em determinados momentos da fermentação pode ser de grande interesse para definir mecanismos e gerar hipóteses pressupondo o que está ocorrendo em determinado momento dentro das células.

Uma das formas de fazer isso é através da técnica de PCR de transcrição reversa quantitativa em tempo real (qRT-PCR), que verifica a expressão de determinados genes. Este processo resumidamente inclui extração de RNA, sintetização de cDNA, utilização de nucleotídeos polimerizantes selecionados (primers com genes alvo), um corante fluorescente e coadjuvantes de reação. Os corantes normalmente utilizados podem ser do tipo sonda fluorogênica TaqMan ou SYBR Green I (Heid et al., 1996; Morrison et al., 1998), atualmente utilizados na forma de mistura principal (Master-mix) contendo junto do corante enzimas DNA-polimerase, solução salina tamponada e solução de magnésio (Raso and Biassoni, 2020). Com um termociclador específico (*Real-Time PCR*), os dados de fluorescência bruta são coletados, ajustados e manipulados para gerar os dados de saída utilizados para análise (Wong and Medrano, 2005).

2.5 Métodos de avaliação e comparação ao longo do tempo

2.5.1 Marcadores do envelhecimento sobre as borras

Os vinhos espumantes contêm uma grande variedade de compostos orgânicos como proteínas, peptídeos, polissacarídeos, monossacarídeos, lipídios, ácidos graxos, ácidos nucleicos, entre outros, e muitos deles (ou seus precursores) são originários das uvas e em menores quantidades, das leveduras (Carrascosa et al., 2011). Muitos dos compostos liberados pelas leveduras estão correlacionados com a autólise celular, que consiste na degradação de constituintes celulares pela ação de enzimas hidrolíticas após a morte celular, durante o envelhecimento do vinho sobre as borras (Alexandre, 2019). Durante este período de maturação do vinho, onde não há mais fonte de nutrientes disponível, as leveduras podem entrar em processos autofágicos, morrer, passando por processos bioquímicos endógenos que catalisam a degradação de biopolímeros intrínsecos das leveduras, resultando em uma excreção substancial de compostos altamente variáveis, como por exemplo, peptídeos, aminoácidos, manoproteínas, glucanos, lipídios, nucleotídeos, entre outros componentes intracelulares que aumentam a complexidade do vinho (Alexandre and Guilloux-Benatier, 2006).

A evolução dos compostos nitrogenados é caracterizada pela liberação inicial de peptídeos de maior peso molecular, que são posteriormente degradados à medida que a maturação se

prolonga, resultando no acúmulo de aminoácidos livres, representando um marcador valioso para seguir a evolução dos processos de autólise das leveduras no envelhecimento dos espumantes (Fiechter and Mayer, 2011).

Proteínas e polissacarídeos são macromoléculas com importante papel na qualidade do vinho. A faixa de concentração da fração proteica no vinho branco é ampla, variando de 30 a 230 mg/L (Ferreira et al., 2001) e seu peso molecular pode variar de, de 6 a 200 kDa (Santoro, 1995). Dependendo de sua concentração, a sua desnaturação pode causar instabilidade proteica e turvar os vinhos (Cosme et al., 2020). Sobre os polissacarídeos, especificamente glucanos e manoproteínas, foi descrito que eles possuem a capacidade de melhoram as propriedades dos espumantes contribuindo na estabilidade da espuma (Martínez-Lapuente et al., 2015), aumento da sensação de volume de boca e diminuição da adstringência (Escot et al., 2001; González-Royo et al., 2013).

2.5.2 Compostos voláteis em espumantes

Muitos dos aromas encontrados nos vinhos são provenientes das próprias uvas (Bakker and Clarke, 2011), porém os aromas formados durante a fermentação pelas leveduras também tem um papel muito importante na composição aromática global (Pretorius, 2016). Todas as bebidas alcoólicas fermentadas são ricas não somente em álcool etílico, mas também em outros metabólitos importantes da fermentação alcoólica, como é caso dos álcoois superiores, ácidos graxos e os seus ésteres. Os álcoois superiores são principalmente de origem fermentativa e estão presentes em doses que variam de 150 a 550 mg/L. Esses álcoois, como os posteriores ésteres formados por eles, possuem um odor intenso podendo ter um importante papel no aroma dos vinhos (Ribéreau-Gayon et al., 2021). Por exemplo, o hexanol tem um aroma descrito como grama verde, ervas, e tem um sabor de “vegetal”, o 2-feniletanol que tem como descritor aromático rosas é considerado um dos álcoois aromáticos mais importantes para a qualidade sensorial de vinho brancos (Bakker and Clarke, 2011).

Os ésteres etílicos (ésteres dos ácidos graxos) e os acetatos (grupo funcional derivado do ácido acético) constituem uma parte dos aromas fermentativos de tipo frutado e floral e representam o maior grupo de compostos com impacto organolépticos produzidos durante a fermentação alcoólica. O frescor e os aromas frutados de vinhos jovens derivam, em grande parte, da presença desses ésteres produzidos durante a fermentação, como por exemplo, o acetato de isoamila que atribui o aroma de banana ou acetato de 2-feniletil que atribui o aroma de rosas (Bakker and Clarke, 2011; Lambrechts and Pretorius, 2000).

Nas fermentações vínicas, através do metabolismo das leveduras e reações químicas entre álcoois e ácidos, são formados uma quantidade muito grande de ésteres. Os acetatos de álcoois superiores estão entre os principais responsável pelos aromas de fermentação de vinhos brancos e são sintetizados pelas leveduras através da reação de acetil-CoA com álcool, catalisada pela enzima álcool acetiltransferase (Saerens et al., 2008). Os ésteres etílicos são os mais abundantes devido à reatividade dos álcoois primários e à quantidade de etanol muito maior que outros álcoois. Resumindo, os processos fermentativos aportam esterificações de natureza enzimática e a conservação dos vinhos aporta esterificações de natureza química (Ribéreau-Gayon et al., 2006b). Os ácidos graxos voláteis, como o ácido hexanóico, octanóico e decanóico também são formados pelo metabolismo de leveduras (a partir de ácidos graxos saturados). Todos esses compostos, dependendo da concentração, influenciam negativamente os aromas dos vinhos (Barbosa et al., 2009).

Em vinhos e outras bebidas fermentadas, uma das formas de avaliar a composição volátil dos vinhos é utilizando microextração em fase sólida (SPME) com fibra de poliacrilato pelo modo *headspace* junto com cromatografia de gás acoplada à espectrometria de massas (CG/EM). Nessa técnica normalmente é utilizado um padrão interno que serve como referência para a quantificação das área dos picos do cromatograma (Schwarz et al., 2020). As vantagens dessa técnica é que normalmente se utilizam pequenos volumes de amostra, é realizada de forma simples e rápida e não requer extração de solvente e padrões individuais de cada composto. Porém, a técnica exige cuidados relativos às condições de preparação das amostras, extração dos analitos e posterior análise cromatográfica que deve ser definida caso a caso em função das características físico-químicas da amostra (Rocha et al., 2001).

2.5.3 Análise sensorial

Análise sensorial é considerada uma ferramenta científica que pode definir objetivamente as características sensoriais dos produtos, a fim de obter informações específicas para expressar e caracterizar determinado produto. Além disso, as técnicas e métodos utilizados nas análises sensoriais podem ser usadas para discriminar amostras, testar preferências hedônicas, caracterizar e quantificar compostos (Mignani et al., 2019). Na enologia os métodos de análise sensorial são amplamente utilizados tanto durante os processos de elaboração, como após os produtos já estarem prontos (Bailetti et al., 2018; de Matos et al., 2020; Sánchez-Palomo et al., 2019, 2017; Ubeda et al., 2019; Wang et al., 2016).

Dentre os testes discriminativos de escolha forçada, teste triangular é um dos mais robustos. Neste teste os julgadores recebem 3 amostras simultaneamente ordenadas por um

protocolo com delineamento balanceado e aleatório, onde, duas amostras são iguais e uma é diferente. Os resultados do teste triangular assumem uma distribuição binomial (0, o provador não identificou corretamente a amostra diferente; 1, o provador identificou corretamente a amostra diferente). Normalmente esse teste é utilizado para determinar se existe diferenças ou similaridades perceptíveis entre amostras. Essa diferença pode envolver um ou vários atributos sensoriais, no entanto não mede a direção nem magnitude dessa diferença. É um método eficaz para determinar se uma alteração no ingrediente, processamento, embalagem ou armazenamento resultou em diferenças no produto. O método do teste e todos os detalhes de aplicação e interpretação dos resultados são normatizados pela ISO 4120 (ISO, 2004).

Já entre os métodos de análise sensorial descritivos, que normalmente englobam descrever e quantificar características dos produtos, a análise descritiva quantitativa (ADQ) é o método mais conhecido e amplamente utilizado, onde um painel treinado é usado para identificar atributos qualitativos e quantitativos de um produto (Bailetti et al., 2018). Esse é o método sensorial mais abrangente, flexível e útil, proporcionando informações detalhadas de um determinado produto (Murray et al., 2001). Procedimentos e detalhes desse método estão descrito na norma IS0 8586 (ISO, 2012), além de detalhes sobre a seleção e os procedimentos para o treinamento e o monitoramento dos avaliadores.

3 Objetivos

3.1 Objetivos Gerais

Verificar o comportamento das leveduras durante a fase inicial de adaptação, fermentação e pós-fermentação, bem como o efeito da presença de SO_2 e etanol, diante de diferentes condições ambientais na elaboração de espumantes, a fim de determinar em quais condições e em que períodos ocorrem níveis mais elevados de estresse. Além disso, comparar se os métodos de elaboração Charmat e Tradicional causam interferências significativas no comportamento das leveduras e nas características dos espumantes ao longo do tempo de envelhecimento em contato com as borras.

3.2 Objetivos específicos

• Testar se a presença de etanol e SO_2 no início da fermentação aumenta o estresse das leveduras e interfere na vitalidade e viabilidade.

• Avaliar o comportamento das populações de leveduras durante a segunda fermentação e envelhecimento de espumantes elaborados pelos métodos Tradicional e Charmat.

• Avaliar e comparar a vitalidade, viabilidade e o comportamento autofágico das leveduras durante a segunda fermentação de espumantes elaborados pelos métodos Tradicional e Charmat por meio da expressão de genes específicos e marcadores enzimáticos.

• Avaliar a liberação de compostos relacionados ao envelhecimento do vinho com as borras das leveduras durante a segunda fermentação de espumantes elaborados pelos métodos Tradicional e Charmat.

• Determinar e comparar os compostos voláteis presentes nos vinhos espumantes elaborados pelos métodos Tradicional e Charmat.

• Comparar os métodos Tradicional e Charmat por meio de uma análise sensorial discriminativa e quantitativa.

4 Resultados

Os resultados da Tese estão divididos em três capítulos e seis apêndices. Os capítulos são apresentados na forma de artigos, cada um com objetivos específicos, porém todos relacionados com elaboração de espumantes. O tema do capítulo 1 aborda o início da segunda fermentação de espumantes. Esse momento é considerado delicado e as características químicas do vinho base podem ser um fator de estresse para as leveduras. Mostramos nesse capítulo como o sinergismo entre o SO_2 e o etanol pode ser prejudicial as leveduras e consequentemente, o sucesso da segunda fermentação dos espumantes. Os trabalhos nos apêndices II e III (artigos técnicos) abordam o mesmo tema e ampliam a discussão. No capítulo 2 realizamos uma comparação do comportamento das leveduras durante a segunda fermentação e envelhecimento dos espumantes elaborados pelos métodos Charmat e Tradicional. Devido as peculiaridades de cada método o comportamento das leveduras é diferente. Os resultados implicam em uma série de questões relacionadas com sobrevivência e morte celular e possibilitam a continuidade de estudos relacionados com esse tema. No 3 capítulo, acompanhamos ao longo do tempo os espumantes elaborados pelos métodos Charmat e Tradicional realizando análises da composição volátil e análises sensoriais em diversos momentos ao longo de aproximadamente 2 anos. De maneira geral, os resultados mostram que durante esse período os espumantes obtidos pelos dois métodos são similares.

Nos apêndices IV, V e VI são apresados os resumos referentes aos estudos realizados durante o período do programa CAPES, PDSE (Programa de Doutorado Sanduíche no Exterior) edital 2022. O programa foi realizado na *Fondazione Edmund Mach - San Michele All'Adige*, situada na cidade de *San Michele all'Adige* (TN) na Itália, durante o período de 01/09/2022 - 01/03/2023. No apêndice IV o trabalho trata sobre a modulação aromática de espumantes *Prosecco* a partir do utilizo de diferentes leveduras e nutrientes durante a elaboração de vinhos base obtidos a partir da variedade de uva Glera. No apêndice V e VI o tema dos estudos estão relacionados com o utilizo de variedades resistentes a doenças (PIWI – *pilzwiderstandsfähig*) para elaboração de espumantes. Nestes estudos os resultados são promissores e apontam uma alternativa para a modulação aromática dos espumantes e para uma vitivinicultura mais sustentável também nessa tipologia de produto.

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4.1 Capítulo 1

Yeast stress and death caused by the synergistic effect of ethanol and SO_2 during the second fermentation of sparkling wines

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ABSTRACT

Problems can often arise at the beginning of the second fermentation (*prise de mousse*) of sparkling wines, such as no start, a long lag period or slow fermentation. These problems are generally associated with yeast stress when inoculated in a base wine with high ethanol content and low pH. However, few studies focus on sulphites, which are often added to base wines to prevent malolactic fermentation, microbiological instability, and wine oxidation. This study aimed to evaluate the joint effect of ethanol and sulfur dioxide on yeasts during the second fermentation. For this purpose, yeasts (*Saccharomyces cerevisiae* EC1118) were subjected to ethanol, sulfur dioxide and ethanol/sulfur dioxide at the beginning of fermentation, and their vitality and viability, as well as the accumulation of intracellular reactive oxygen species and intracellular pH, were evaluated by flow cytometry. Furthermore, the expression of genes involved in sulfur transport and metabolism was determined. The results showed high mortality, ROS accumulation and intracellular pH reduction in fermentations with both ethanol and sulfur dioxide. The negative effect of ethanol, sulfur dioxide and ethanol/sulfur dioxide on yeasts was found to be dose-dependent and high in those commonly found in some base wines. Cells treated with ethanol/sulfur dioxide showed over-expression of genes involved in sulphite transport (*SUL1* and *SUL2*), efflux pump (*SSU1* and *FZF1*) and metabolism of sulfur amino acids (*MET14*). Altogether, our data indicate that ethanol and sulfur dioxide have a synergistic effect on yeasts, which may be the root cause of the problems encountered at the beginning of the second fermentation of sparkling wines, and should thus be seriously taken into consideration by winemakers.

KEYWORDS

vitality, viability, ROS, intracellular pH, gene expression

INTRODUCTION

Sparkling wines develop over two consecutive fermentations: the first one converts grape must into base wine, and the second favours CO_2 incorporation (Di Gianvito *et al*., 2019). The base wines are characterised by an alcohol content of 9.5 % - 11.5 % (v/v) and relatively higher acidity (compared to still wines), with a pH index of 2.8 - 3.3 (Togores, 2018). The second alcoholic fermentation or *"prise de mousse"* (literally "foam creation"), is carried out in closed vessels (hermetic tanks or bottles) causing the incorporation of carbon dioxide into the liquid (Carrascosa *et al*., 2011). This second fermentation can be performed using either the traditional method (fermentation in the bottle) or the Charmat method (fermentation in a tank). For this purpose, together with the base wine, a mix (*liqueur de tirage*) is added with a specific amount of sugars per litre of wine (usually sucrose cane or beet sugar at 20 - 25 g/L) and yeast starter culture for the fermentation (Di Gianvito *et al*., 2019).

The chemical composition of the base wine and the conditions of the second fermentation usually stressing factors for the yeast inoculum: for example, high ethanol concentration, presence of glycerol and low pH (Borrull *et al*., 2015), presence of sulfur dioxide (SO_2) (Sudraud *et al*., 1985), presence of acetic acid (Giannattasio *et al*., 2013), relatively low fermentation temperatures and concentration of nutrients (Kemp *et al*., 2020; Martí-Raga *et al*., 2015), and presence of endogenous CO_2 (Porras-Agüera *et al*., 2020). Therefore, the adaptation of the yeasts to the base wine is important for the success of the second fermentation (Benucci *et al*., 2016; Borrull *et al*., 2016; Martí-Raga *et al*., 2015). This adaptation procedure is known as *pied-de-cuve* (in the French language) and consists of two important phases: adaptation to the alcoholic medium and active growing phase (Benucci *et al*., 2016). This process can last for several hours or days, and usually with a gradually increasing concentration of ethanol and sugar (Benucci *et al*., 2016; Borrull *et al*., 2016; Martí-Raga *et al*., 2016). Traditional yeast adaptation and inoculation protocols for the second fermentation recommend a final inoculum of approximately 1.5 x 10^6 cells/mL of base wine (Ribéreau-Gayon *et al*., 2006). According to these authors, it is possible for levels well below this to cause sluggish fermentations and leave unfermented sugars. Conversely, higher levels (above 2 x 10^6 cells/mL) speed up fermentations; however, depending on the strain, they can result in excessive yeast taste in the final product due to yeast autolysis.

Wine is a hydroalcoholic solution in which ethanol is the second most abundant compound (Waterhouse *et al*., 2016) and an inhibitor for several microorganisms. In the conditions of the second fermentation of sparkling wines, ethanol is described as the main environmental factor to have an influence on yeast transcriptional responses (Penacho *et al*., 2012). Among several factors tested in a study by, ethanol in the base wine was considered to be the main stress factor for yeasts in the second fermentation of sparkling wines. Tolerance to ethanol varies widely among yeast species (Lin *et al*., 2020), and within the *Saccharomyces cerevisiae* species, tolerance may vary depending on the strain (Borrull *et al*., 2015). The presence of ethanol can cause structural changes in yeasts cells, which can impact the fluidity of the plasmatic membrane (Navarro-Tapia *et al*., 2018), and consequently cell morphology (Dinh *et al*., 2008).

Another important compound pointed out as being a stress factor in base wines is SO_2, which is used as a preservative in these wines until the second alcoholic fermentation. Sulfur dioxide is added to grape musts and wines to reduce the medium (antioxidant activity) and to inhibit undesirable microorganisms (antimicrobial action) (Blouin and Peynaud, 2006; Gould and Russell, 2003). Although the use of SO_2 for the conservation of wines is an old practice (Gould and Russell, 2003), ingestion of sulphites through the consumption of food and drink can cause some related adverse clinical effects (Vally *et al*., 2009) ; for this reason, there is a worldwide movement towards decreasing the concentration of sulphites in wines. Although several studies have shown alternatives for SO_2 and sought to reduce its use (Capece *et al*., 2020; Christofi *et al*., 2021; Marchante *et al*., 2019; Shih *et al*., 2020; Simonin *et al*., 2020; Zara and Nardi, 2021), to date, no other physical technique or chemical additive can provide the efficacy and broad spectrum of action of this compound (Lisanti *et al*., 2019).

The microbial inhibition by SO_2 in yeasts has been attributed to several cellular changes, such as the modification of membrane transport activity by binding to membrane proteins (Divol *et al*., 2012); the inhibition of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) - a critical enzyme in the glycolysis pathway (Hinze and Holzer, 1986) - and other enzymes like ATPase,

alcohol dehydrogenase and NAD-glutamate dehydrogenase (Maier *et al*., 1986), causing a decrease of the ATP content in cells (Hinze and Holzer, 1986; Maier *et al*., 1986; Schimz and Holzer, 1979); the modification of the expression of many genes correlated with cell metabolism (Park and Hwang, 2008); the degradation of available thiamine (Labuschagne and Divol, 2021); the binding of metabolites (acetaldehyde, pyruvate, glucose, dihydroxyacetone-phosphate, oxaloacetic acid, and α-ketoglutaric acid), thereby preventing their further use as substrates for metabolic pathways (Rankine and Pocock, 1969). However, wine yeasts, particularly *S. cerevisiae*, have a certain tolerance to SO_2 (García-Ríos and Guillamón, 2019) and this tolerance varies between strains (Nadai *et al*., 2016). SO_2 tolerance is a desired trait in wine yeasts which has been unconsciously selected in wine-making practices over time (Zimmer *et al*., 2014), and can be considered an evolutionary advantage (García-Ríos *et al*., 2019).

To reduce the toxicity of sulfur dioxide, yeasts use several mechanisms, such as increasing the production of acetaldehyde to bond with SO_2 and thus reducing the free fraction (Cheraiti *et al*., 2010; Park and Hwang, 2008); activating the Ssu1p sulphite pump encoded with the *SSU1* gene (Marullo *et al*., 2020; Zara and Nardi, 2021); activating sulfur amino acid biosynthesis (Divol *et al*., 2012) ; and/or modifying the overall metabolic and cell cycle that lead to a "viable but non-culturable" cell behaviour (Divol and Lonvaud-Funel, 2005; Salma *et al*., 2013).

The effect of ethanol and sulfur dioxide on the individual antimicrobial action and resistance to stress of wine yeast of the *Saccharomyces cerevisiae* species has been studied in the past decades; however, relatively few studies discuss the joint effect of these compounds on yeast cells (Chandra *et al*., 2015). Long lag phases and lazy fermentations during the second fermentation of sparkling wines are often reported in the industry. The results of a study on the preparation of starter cultures (*pied de cuve*) for sparkling wine production indicate that differences in fermentative kinetics may be caused by differences in the content of ethanol and SO_2 in base wines (Benucci *et al*., 2016). As reported in spoiling wine yeasts (Chandra *et al*., 2014; 2015; Edwards and Oswald, 2018), the presence of ethanol and SO_2 can modify yeast behaviour and viability.

The second fermentation of sparkling wines is a delicate step in the process, which can impact the final product. Predicting the progress of this step and any problems that may occur can be crucial for obtaining a quality sparkling wine. Many empirical reports express the concern of winemakers regarding this initial stage of the process, as well as their lack of full and sound understanding of what causes viability reduction and the increase in lag phase. For these reasons, we decided to explore the topic more deeply by determining whether the presence of both ethanol and SO_2 molecules causes synergism to the extent of modifying homeostasis and physiological stress responses in yeasts during inoculation and at the beginning of the second alcoholic fermentation of sparkling wines.

MATERIALS AND METHODS

The *Saccharomyces cerevisiae* yeast strain Lalvin EC-1118® (Lallemand, Canada) was used in all assays. This yeast strain is recommended and conventionally used in the second fermentation of sparkling wines. Moreover, its genome is already sequenced (Novo *et al*., 2009), a factor that facilitated molecular analyses.

1. Experimental designs

1.1. Synthetic wine and inoculum preparation

All experiments evaluating the synergism between SO_2 and ethanol were conducted in a synthetic wine (Martí-Raga *et al*., 2016) with 4 g/L tartaric acid, 0.5 g/L citric acid, 0.5 g/L malic acid, 0.134 g/L sodium acetate, 1.7 g/L YNB (with ammonium sulfate and without amino acids) and 4.0 g/L glycerol. The final pH of the solution was adjusted to 3.1 using sodium hydroxide. Different ethanol and sulfur dioxide concentrations were added to this medium following the experimental design.

For the adaptation of yeast (*pied de cuve*) a modified protocol was followed (Benucci *et al*., 2016). Briefly, a yeast colony (Lalvin EC-1118) was added to the YPD broth (2 % yeast extract, 2 % glucose, 1 % peptone, pH 6.5) and grown and shaken (150 rpm) at 28 °C for 20 hr. Next, for the adaptation of the inoculum, 10 % (v/v) ethanol, 50 g/L of sucrose and 3.5 g/L of dibasic ammonium phosphate were added to the synthetic wine. An equal volume of the base wine was added to the initial culture (1:1 v/v) and kept static at 20 °C for 24 h. This culture was then diluted with the synthetic wine to a ratio of 1:3 and maintained at a temperature of 20 °C for another 24 h. After this, in all treatments, 24 g/L of sucrose

(for fermentation) was added to the synthetic wine and inoculated with 1.4 x 10^6 cells/mL of adapted yeasts.

1.2. The effect of ethanol and SO_2 during fermentation

The treatments were divided into four groups in triplicate: i) no EtOH or SO_2 (the control), ii) with 20 mg/L SO_2 (from potassium metabisulphite solution), iii) with 10 % v/v EtOH, and iv) with 20 mg/L SO_2 + 10 % (v/v) EtOH. The fermentations were monitored at a controlled temperature of 15 °C for 28 days (Figure 1). In this experiment, the yeast growth and viability were microscopically monitored as described in Section 2.1.

1.3. Evaluation of stress markers

The treatments were applied as previously described. In this experiment, the fermentations were also carried out under a controlled temperature of 15 °C for 6 days (Figure 2). Yeast growth and viability were determined by microscopic methods and colony-forming units (CFU) evaluated on YPD agar (methods described in Section 2.1.). Cell membrane integrity, intracellular ROS and intracellular pH were analysed by flow cytometry as described in Section 2.2.

1.4. Treatments with different concentrations of ethanol and SO_2

In this experiment, the treatments were divided into eight groups in duplicate: i) no added EtOH or SO_2 (the control), ii) 10 mg/L SO_2, iii) 20 mg/L SO_2, iv) 10 % EtOH, v) 10 % EtOH + 10 mg/L SO_2, vi) 5 % EtOH + 20 mg/L SO_2, vii) 10 % EtOH + 20 mg/L SO_2, and viii) 12 % EtOH + 20 mg/L SO_2. All the treatments were inoculated with 1.4 x 10^6 cells/mL of adapted yeasts. The fermentation temperature was held at 20 °C for 96 h (Figures 3, 4, 5 and 6; Table 2). The cell membrane integrity/cellular vitality, intracellular ROS, intracellular pH, concentrations of reducing sugars, free SO_2 and acetaldehyde were evaluated as described in Sections 2.2. and 2.3. Gene expression was also quantified (see Section 2.4. for the method) and related to the presence of sulfur dioxide in the fermentation environment at the sample collection points closest to the time of inoculation (after 12 h and 24 h).

1.5. Second fermentation on an industrial scale

In the second industrial scale fermentation (traditional method), the base wine comprised a blend (*assemblage*) of wines from the Chardonnay grape varieties (36 %), Riesling Italic (30 %), and Pinot Noir (34 %) (white vinified). The wine alcohol concentration was 11.2 % v/v, and it had a pH of 3.27, 80 mg/L of total SO_2 and 16.5 mg/L of free SO_2. Approximately 22 g/L of sucrose (*liqueur de tirage*) and an adapted inoculum were gradually added to the base wine following a specific company protocol. The percentage of ethanol in the inoculum at the time of inoculation was 13.3 % (v/v) and the total yeast population was 3.5 x 10^7 cells/mL with a viability of approximately 70 %. The base wine was inoculated with 1.4 x 10^6 cells/mL; that is 4 % (v/v) of the final fermentation volume. The bottles were kept at 12 °C for 21 days and three bottles were analysed weekly. The cell membrane integrity/cellular vitality and free SO_2 were analysed (methods described in Sections 2.2. and 2.3.).

2. Analyses performed

2.1. Yeast growth and viability assays

Growth and viability of yeast cells (exclusion tests) were performed using a light microscope (Olympus IX71) with a x 400 magnification using a Neubauer chamber, and viability was determined by staining with a 0.1 % Trypan Blue solution (Thermo Fisher Scientific, MA, EUA) (McGahon *et al*., 1995). Moreover, viable and culturable cells were determined by serial dilution, plating and colony counting on YPD agar (1 % yeast extract, 2 % peptone, 2 % glucose and 2 % agar). The plates were incubated at 28 °C for 24 h, the colony units were counted and data expressed as colony-forming units (CFU/mL) (Fugelsang and Edwards, 2007).

2.2. Flow cytometer analyses

To carry out the analyses with the flow cytometer, samples were centrifuged to separate the cells (4629 x g for 5 min). Once separated, the yeasts were washed in phosphate buffered saline with a pH of 7.2 (PBS) and stained with specific fluorescent dyes.

Flow cytometry analyses were performed in a FACSCalibur flow cytometer (Becton-Dickinson, CA, USA) equipped with an argon-ion laser emitting at 488 nm. The flow cytometer data of 20,000 cells were acquired using CellQuest Pro software (BD Bioscience) and data analysis was carried out using FlowJo v.10 software (TreeStar, Inc). All samples were incubated for 30 min in the dark before analysis.

The cellular vitality and cell membrane integrity were determined using the LIVE/DEAD™ FungaLight™ Yeast Viability Kit (Thermo Fisher Scientific), which includes 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA) - cleaved by nonspecific esterases resulting in a fluorescent product - and propidium iodide (PI), which only penetrates membrane damaged cells. The staining and flow cytometry analyses were performed according to the manufacturer's recommendations.

The intracellular ROS (Reactive Oxygen Species) was analysed with 2′-7′-dichlorofluorescein diacetate (DCFH-DA) (Sigma, MO, USA). A two-electron oxidation of DCFH-DA results in the formation of a fluorescent product, dichlorofluorescein (DCF) (Kalyanaraman *et al*., 2012) ; the fluorescence intensity of DCF is related to the amount of ROS within the cells. Stock solutions were prepared by dissolving DCFH at 5 mg/ml in DMSO (dimethylsulfoxide). Staining was performed in 500 µl of sample and 5 µg/ml of dye solution.

The intracellular pH was determined using the fluorescent probe pHrodo Green AM Intracellular pH Indicator (Thermo Fisher Scientific). The pHrodo Green is slightly fluorescent at neutral pH and fluorescent in acid conditions. The sample preparation procedure was performed according to the manufacturer's recommendations. The results were expressed in relative fluorescence, because, due to the low pH of the wines, part of the treatments remained outside the ideal range of pH variation detectable by pHrodo Green (pH 9-4).

The sizes of the cells were compared using forward scatter measurement (FSC); the intensity of the FSC is proportional to the diameter of the cell and is mainly due to the diffraction of light around the cell (Leif, 1986).

2.3. Physicochemical analyses

These analyses were performed together from samples of the supernatant (samples centrifuged 4629 x g for 5 min) which had been frozen at the time point of each collection. Total reducing sugars (g/L) were evaluated via the hydrolysis of sucrose in an acid medium and colorimetric method using 3,5-dinitrosalicylic acid (DNS) and microplate reader (absorbance 595 nm) (Dos Santos *et al*., 2017). The acetaldehyde (ethanal) concentration (mg/L) was quantified using the colorimetric method (acetaldehyde reacts with sodium nitroferricyanide and piperidine solution) using a spectrophotometer (570 nm absorbance), and the calibration curve was obtained directly using acetaldehyde in different concentrations, as adapted from the OIV method (OIV, 2009). Free SO_2 (mg/L) was estimated according to the Ripper titrimetric method using iodate (Zoecklein *et al*., 1999).

2.4. Gene expression

RNA extraction was performed according to a protocol specific for *Saccharomyces cerevisiae* (Shedlovskiy *et al*., 2017). The extracted RNA was treated with DNase I (Thermo Fisher Scientific) following the manufacturer's protocol. The absence of contaminant genomic DNA in the RNA was checked before cDNA synthesis using RNA as a template for a PCR assay. The RNA was reversed-transcribed into cDNA with the M-MLV Reverse Transcriptase (Thermo Fisher Scientific) following the manufacturer's protocol. The primers for qRT-PCR are shown in Table 1. Their sequences were obtained from published studies and by using the Primer designing tool system on the NCBI website (Primer-BLAST). Primers were purchased from Thermo Fisher Scientific.

Real-time PCR was performed in Applied Biosystems StepOne qRT-PCR (Thermo Fisher Scientific) using SYBR Green as a fluorophore. Reactions were carried out in 20 µL of mix containing 10 µL of Platinum ™ SYBR ™ Green qPCR SuperMix-UDG dye (Thermo Fisher Scientific), 2.0 µL of primer mix (200 nM final concentration), and 8 µL of cDNA. The thermocycling programme consisted of one hold at 95 °C for 3 min, followed by 40 cycles of 15 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C. After the cycles, melting-curves data were collected to verify PCR specificity and contamination. Moreover, in an initial experiment, three potential housekeeping genes were evaluated: *ACT1*, *TEF1,* and *IPP1*. As all of them gave similar Ct values for the different samples, *ACT1* was selected for further analysis.

With the values obtained from the expression of each gene in the treatments, ΔCт was calculated by subtracting C_T (threshold cycle) of the reference gene (*ACT1*) from C_T of the target gene. Afterwards, ΔΔCт was calculated [ΔC_T (target sample) - ΔC_T (sample reference)], subtracting the ΔCт of the genes from the treatment samples (20 mg/L SO_2 without EtOH, – 10 % EtOH without SO_2, 10 % EtOH + 20 mg/L SO_2) by the control sample (without EtOH and without SO_2). Then the formula for the comparative method was applied

TABLE 1. Genes and primers used in qRT-PCR

Gene	Primer F (5' - 3')	Primer R (5' - 3')	Size (bp)	Function	Reference
ACT1	TCGCCTTGGACTTCGAACAA	CAAAGCTTCTGGGGCTCTGA	128	Housekeeping gene	This paper
IPP1	AGCCAGTTTCTGCCTTCCACGA	TGGTGATTTCTAACTTGGCGTTGGT	111	Housekeeping gene	This paper
TEF1	GGTTACTCTCCAGTTTTGGATTGTC	ACGAACTTGACCAAAGCAGC	149	Housekeeping gene	(Nardi *et al*., 2010b)
COM2	AGCCTTGGTTGTGAACCCAT	GCGTGGTCACTCTCATCACT	185	SO_2 tolerance regulator	This paper
FZF1	CCAGAGAGTTACTGGTCGGATA	CGTGTGGTCATAGTGGTCAAT	102	SO_2 chemical induction	(García-Ríos *et al*., 2019)
MET14	AGAAGTCGCTGAGCAAAGGG	TTCAACCGTCTTCTGGTCGG	145	Sulfur assimilation pathway	This paper
SUL1	CACTGGGTTGGGTATACTGC	ATGAGAGCCGGAATTTGACC	120	Sulphate permease	(Chen *et al*., 2018)
SUL2	AAGGGAGAACGACCCTGAAT	TGGCCTTTCTCAAATCAACC	161	Sulphate permease	(Jennings and Cui, 2012)
SSU1	TTTGCGTTTGTTGGTCAATTCTATGCCTTTTA	TCCACGCTTTCAATGCTGTTATACGGAGAA	151	SO_2 influx of compound	(Nardi *et al*., 2010a)

$(2^{-\Delta\Delta C_T})$ (Livak and Schmittgen, 2001), giving a value for the relative expression of the genes or RQ.

3. Statistical analysis

Statistical analyses were performed by two-way ANOVA and Tukey's multiple comparisons test, using a level of significance of 95 %. Graphs and statistical analyses were performed using the Graphpad Prism® software (GraphPad, CA, USA).

RESULTS

1. The effect of ethanol and SO_2 during fermentation and evaluation of stress markers

In the first experiment, it was possible to verify the impact caused by SO_2 and ethanol on yeast viability and cell growth. As can be observed in Figure 1, yeast cells grown on the control medium showed high viability throughout the experiment and exhibited a typical growth behaviour, attaining the stationary phase with approximately 5 x 10^7 cells/mL after 8 days. On the medium supplemented with SO_2 (20 mg/L), the yeast population underwent a small (approximately 20 %) reduction in viability in the first few days (Figure 1A), which was maintained until the end of fermentation. This initial reduction in yeast viability caused a delay in population growth, but it reached almost the same final cell density as the control (Figure 1B). However, in the medium with EtOH (10 %), cell viability remained high throughout the fermentation, but yeast growth was drastically reduced (Figure 1A and 1B).

Conversely, in the medium containing both EtOH and SO_2 (10 % EtOH + 20 mg/L SO_2), a drastic reduction in yeast viability during the first few days was observed, reaching 2 % on the sixth day (Figure 1A). After this point, the cell population started to grow slowly (Figure 1B), remaining relatively low compared to the control. At the end of the experiment (28 days) the yeast population in EtOH + SO_2 medium was just 1/6 of the control and SO_2 treatments, and 1/2 of the EtOH treatment (Figure 1B). These data indicate that EtOH and SO_2 have a synergistic effect on yeast viability and growth.

To better understand the effect of ethanol and SO_2, and their synergistic effect on yeasts, we conducted a new acute experiment (144 h), monitoring cell viability, vitality, intracellular ROS and intracellular pH. As shown in Figure 2 (A, B, C and D), this experiment confirmed the synergistic effect of ethanol and sulfur dioxide, which together cause a drastic reduction in cell growth and viability. Yeast growth, determined by microscopic cell counting (Figure 2A) and colony-forming units (Figure 2B) showed a considerable reduction in yeast growth in the presence of sulfur dioxide and, in particular ethanol. However, yeast growth was completely inhibited by the presence of both ethanol and sulfur dioxide in the synthetic wines.

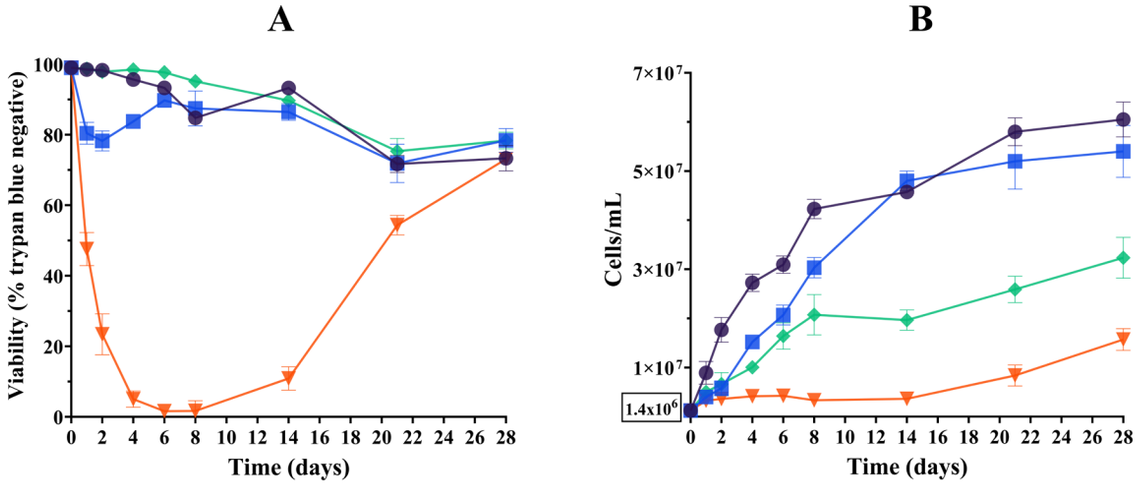

FIGURE 1. Synergism, viability, and growth in synthetic wine. Cell membrane integrity (viability) was assessed with a light microscope and Neubauer chamber, and using trypan blue exclusion dye (A); population growth was estimated using light microscope and Neubauer chamber (B). (●) Control; (■) 20 mg/L SO_2; (■) 10 % (v/v) EtOH; (▼) 10 % EtOH + 20 mg/L SO_2. The error bars in the line graphs represent the standard deviation obtained from triplicate samples within the same experiment.

The cell viability results obtained using trypan blue (Figure 2C) and PI (Figure 2D) showed high viability (> 95 %) in the control- and ethanol-containing medium.

Yeasts cultivated in the medium supplemented with SO_2 exhibited an initial reduction in viability (after 24 and 48 h), followed by an increase in viability associated with population growth. Conversely, yeasts cultivated in EtOH + SO_2 medium showed a drastic and rapid reduction in viability.

The cytometric analysis of intracellular ROS using the DCFH-DA dye showed a basal low ROS concentration for yeast cells grown in the control medium and in the media that contained just ethanol or SO_2.

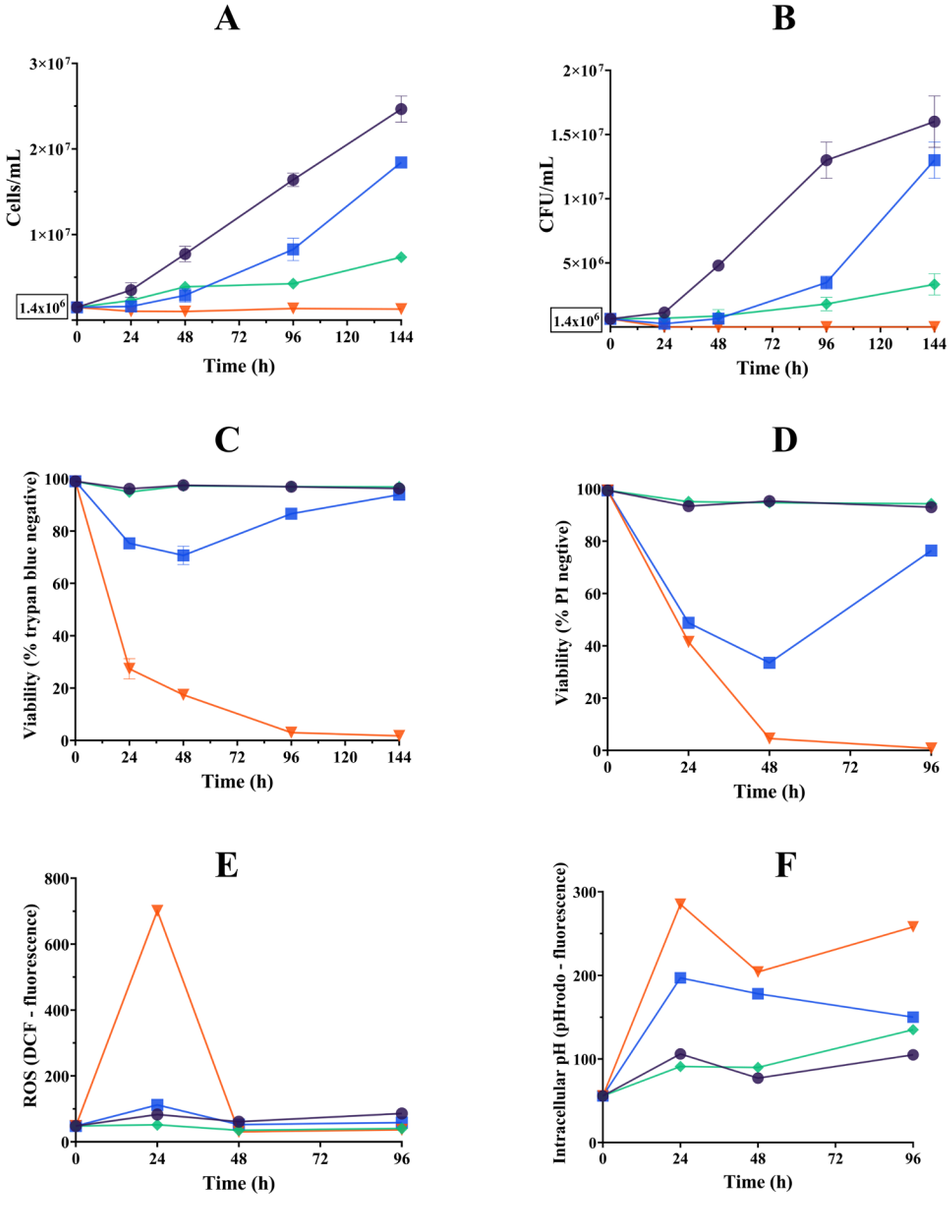

FIGURE 2. Stress caused by synergism between EtOH and SO_2. Estimation of population growth using the light microscope and Neubauer chamber (A); viability test and growth with colony-forming units (CFU) (B); cell membrane integrity (viability) assessed with the light microscope, Neubauer chamber and using trypan blue exclusion dye (C); cell membrane integrity (viability) assessed with flow cytometer + PI (D); intracellular ROS analysed with flow cytometer + DCFH-DA (E); intracellular pH analysed with flow cytometer + pHrodo Green AM (F). (●) Control; (■) 20 mg/L SO_2; (■) 10 % EtOH; (▼) 10 % EtOH + 20 mg/L SO_2. The error bars in the line graphs (A, B and C) represent the standard deviation obtained from triplicate samples within the same experiment.

However, a peak in intracellular ROS occurred on the first day of the treatment supplemented with both ethanol and SO_2 (Figure 2E), suggesting that together these compounds cause greater oxidative stress and may have also influenced the drastic reduction in cell viability.

The analysis of intracellular pH and treatments with SO_2 and with EtOH + SO_2 revealed considerable changes in the fluorescence of the dye (the higher the fluorescence, the lower the pH). However, the EtOH + SO_2 treatment exhibited the highest fluorescence (lower pH) throughout the experiment. This corroborates the results of the other analyses (Figure 2) which showed that the treatment with both molecules intensified the stress in yeast cells, indicating a synergistic effect had taken place. At the end of the experiment, the SO_2 treatment contained 5 mg/L of free SO_2, compared to 10 mg/L in the EtOH + SO_2 treatment.

2. The interaction of different concentrations of ethanol and sulfur dioxide concentrations in yeasts

The control, 10 mg/L SO_2, 10 % EtOH and 10 % EtOH +10 mg/L SO_2 treatments showed almost the same behaviour for all variables, except for sugar consumption (Figure 3B), with the presence of ethanol decreasing fermentation rate.

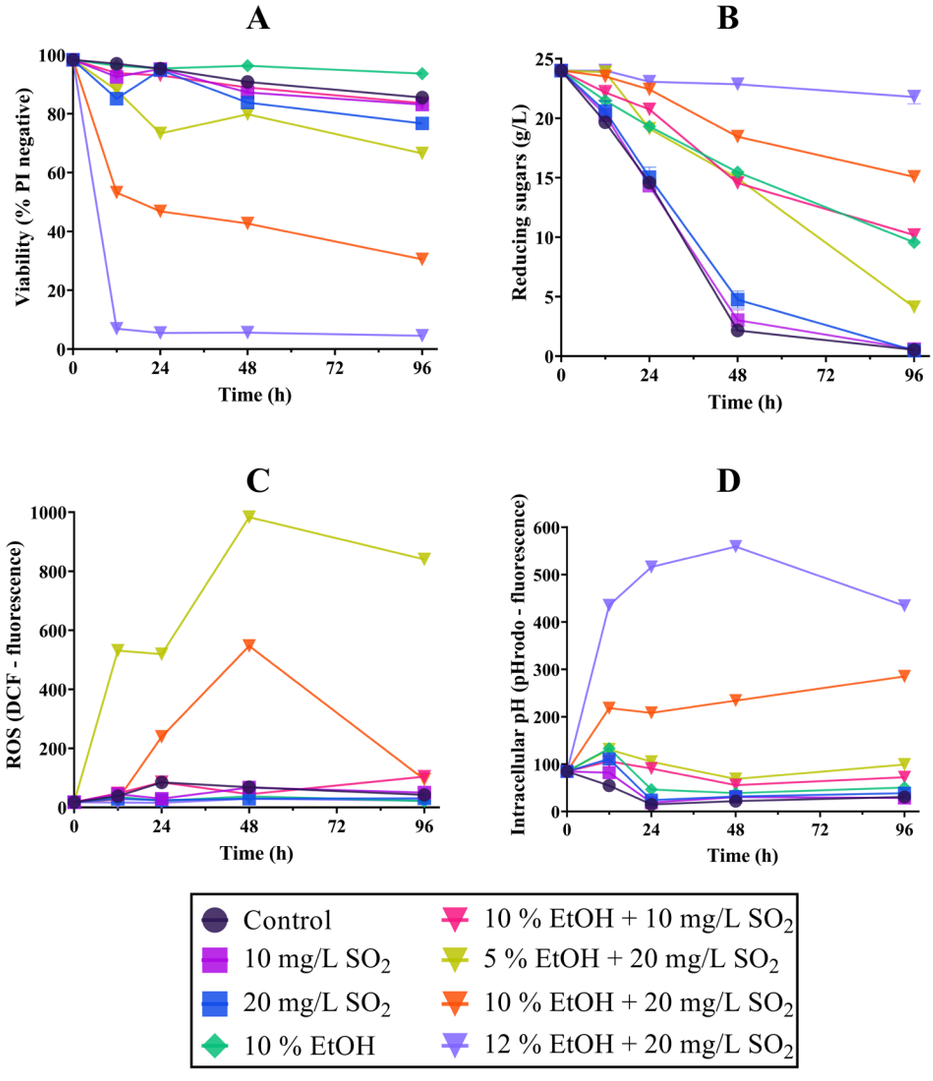

FIGURE 3. Different concentrations of EtOH and SO_2. Cell membrane integrity (viability) assessed with flow cytometer + PI (A); monitoring of consumption of total reducing sugars (B); intracellular ROS analysed with flow cytometer + DCFH-DA (C); intracellular pH analysed with flow cytometer + pHrodo Green AM (D). The error bars in the line graphs (B) represent the standard deviation obtained from duplicate samples within the same experiment.

However, treatments that include both ethanol (5 to 12 %) and sulfur dioxide (20 mg/L) exhibited a reduction in fermentation rate and cell viability proportional to the ethanol concentration. As can be observed in Figures 3A and 3B, the treatment with 5 % EtOH + 20 mg/L SO_2 showed a 27 % reduction in yeast viability in the first 24 h, which was maintained until the 96 h time point, resulting in sluggish fermentations. Treatments with 10 % and 12 % ethanol + 20 mg/L of SO_2 showed a remarkable decrease in cell viability in the first 12 h (by 45 and 95 % respectively) and a proportional inhibition of sugar consumption.

Yeast cells grown in 5 % EtOH + 20 mg/L SO_2 and 10 % EtOH + 20 mg/L SO_2 exhibited a considerable increase in intracellular ROS (Figure 3C): In the former treatment, the ROS which had accumulated during the first 48 h remained high until the end of the experiment, while in the latter, ROS showed a constant increase during the first 48 h, followed by a sharp decrease thereafter. Interestingly, the yeasts in the more severe treatment (12 % EtOH + 20 mg/L SO_2) did not accumulate intracellular ROS - a fact that may be related to the high mortality (> 95 %) observed in this treatment - indicating that ROS accumulation depends on metabolic activity.

Regarding the intracellular pH, data in Figure 3D show that yeast cells cultivated in the highest concentrations of EtOH and SO_2 (10 % EtOH + 20 mg/L and 12 % EtOH + 20 mg/L SO_2 showed greater fluorescence (lower pH intracellular) than the control and the other treatments. This decrease seems to be correlated with the percentage of cells with a damaged membrane, which may indicate the entry of a greater amount of SO_2 into the intracellular environment and/or the failure of proton efflux.

The results of the analysis of yeast using two fluorescent markers (CFDA and PI; Figure 4) showed that EtOH (10 %) did not interfere with yeast cell enzyme activity (vitality) immediately after inoculation (12 h), corroborating the data presented in Figures 1A, 2C, 2D and 3A. Conversely, treatments with 20 mg/L of SO_2, and 10 % EtOH + 20 mg/L of SO_2, had completely different behaviour. In the treatment of 20 mg/L of SO_2, initial stress was observed with a drop in vitality (lower CFDA fluorescence) and a small increase in cells with a damaged membrane (PI positive). In the second treatment (10 % EtOH + 20 mg/L SO_2), yeast cells showed four populations distributed in different quadrants, indicating a higher level of stress, with a considerable increase in the number of cells with the damaged membranes (quadrant Q3). Moreover, an increase of just 2 % EtOH (12 % EtOH + 20 mg/L SO_2) resulted in a significantly higher number of cells with membrane disfunction even after a short 12 h exposure (more than 90 %).

The comparison of cell size using direct dispersion measurement (FSC) by flow cytometry analysis (Figure 5) showed that yeast cells in the control and the 10 and 20 mg/L SO_2 treatments exhibited a "normal" distribution, while yeast cells subjected to 10 % EtOH showed a small percentage of larger cells (Figure 5A). The treatments that cause a more marked reduction in yeast viability (10 % EtOH + 20 mg/L SO_2, 12 % EtOH + 20 mg/L SO_2), showed a high percentage of cells with reduced size (Figure 5D), thus indicating modifications having been made to cell permeability and water content or a possible modification to the metabolism of the membrane lipids (phospholipids).

One of the most important compounds involved in sulfur dioxide neutralisation is acetaldehyde, which is produced during alcoholic fermentation and combines with sulfur dioxide, thus reducing the amount of free SO_2. The results of the analyses of free SO_2 and acetaldehyde (Table 2) show that only the treatments with considerable fermentative activity (Figure 3B) showed a large decrease in free SO_2; meanwhile, the treatment with 12 % EtOH + 20 mg/L SO_2, which exhibited a dramatic inactivation of yeast, showed the lowest acetaldehyde production and maintained high levels of free SO_2 throughout the fermentation process. The treatments with SO_2 showed an increase in acetaldehyde concentration (mainly within 12 h) in comparison to the control, as did those with ethanol alone. The treatments with both molecules (10 % EtOH + 10 mg/L SO_2 and 5-10 % EtOH + 20 mg/L SO_2) showed higher concentrations of acetaldehyde than the other treatments.

Yeast responds to a stress factor (in this case SO_2 and ethanol) with a differential expression of genes that contribute to cell viability and homeostasis. As shown in previous figures, in mid- or non-lethal concentrations of ethanol and sulfur dioxide, a yeast population can adapt and grow, albeit slowly. This "adaptation" involves the differential expression of genes, the products of which contribute to yeast cell homeostasis, metabolism and eventual division. In this context, we evaluated the expression of several genes associated with sulfur transport and metabolism. As can be observed in Figure 6A, the *SSU1*,

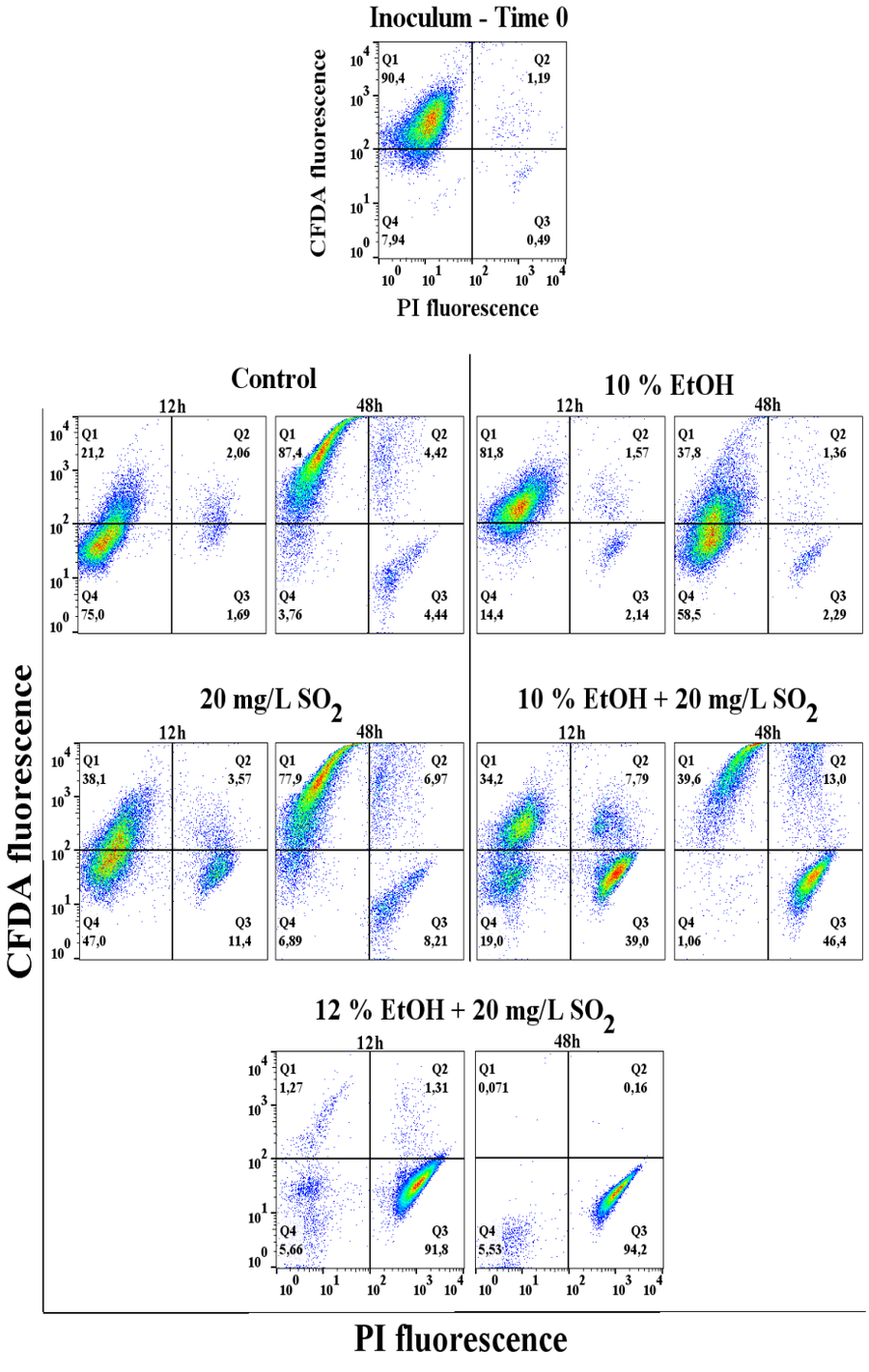

FIGURE 4. Vitality versus viability. Flow cytometer analysis of the third test using the LIVE/DEAD™ FungaLight™ Yeast Viability Kit (CFDA/PI).

MET4, and *FZF1* genes were hyper-expressed in yeasts grown in 10 % EtOH + 20 mg/L SO_2 compared with the treatments that contained just ethanol or SO_2. However, the *SUL1* gene showed higher expression in both 10 % EtOH and 10 % EtOH + 20 mg/L SO_2 treatments, while *SUL2* exhibited higher expression in the treatments that contained SO_2. The *COM2* gene expression did not vary among treatments.

After 24 h (Figure 6B), the yeast cells subjected to 10 % EtOH and 10 % EtOH + 20 mg/L SO_2 showed higher expression of *SSU1* and, in particular, *MET14* genes than the control and the SO_2 treatments. However, the high expression of *SUL1* and *SUL2* observed in the first few hours was not evident after cell adaptation to ethanol and sulfur dioxide stress.

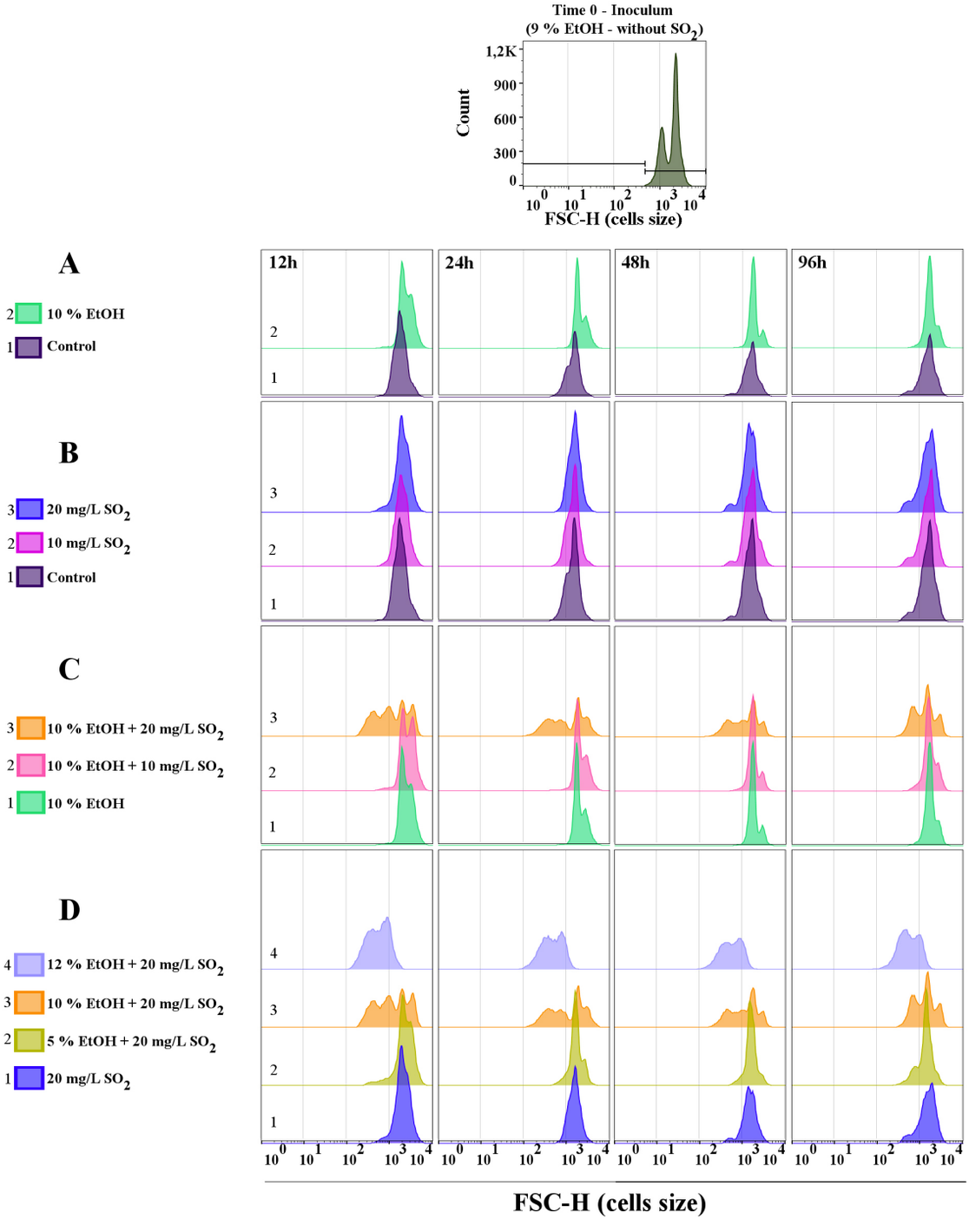

FIGURE 5. Differences in cell size. Analysis was performed with flow cytometry using direct dispersion measurement (FSC). The intensity of the FSC is proportional to the diameter of the cell.

TABLE 2. Analysis of free SO_2 and acetaldehyde over time.

SAMPLE	FREE SO_2 (mg/L)					ACETALDEHYDE (mg/L)			
	Time					Time			
	T0	12 h	24 h	48 h	96 h	12 h	24 h	48 h	96 h
Control	-	-	-	-	-	27.4 ± 2^{ef}	23.3 ± 8^{ce}	45.5 ± 5^{d}	53.8 ± 7^{d}
10 mg/L SO_2	10 ± 0^{A}	3.2 ± 1^{B}	3.2 ± 1^{B}	3.2 ± 1^{B}	3.2 ± 0^{B}	40.8 ± 2^{de}	39.3 ± 2^{c}	52.8 ± 12^{cd}	70.3 ± 1^{cd}
20 mg/L SO_2	20 ± 0^{A}	3.8 ± 0^{B}	3.8 ± 0^{B}	3.2 ± 0^{B}	3.2 ± 0^{B}	62.1 ± 1^{cd}	42.4 ± 3^{c}	64.1 ± 22^{bcd}	78.1 ± 1^{cd}
10 % EtOH	-	-	-	-	-	99.3 ± 5^{b}	68.3 ± 5^{b}	62.1 ± 1^{bc}	78.6 ± 7^{c}
10 % EtOH + 10 mg/L SO_2	10 ± 0^{A}	3.2 ± 1^{B}	3.2 ± 1^{B}	3.2 ± 0^{B}	3.2 ± 0^{B}	125.2 ± 2^{a}	82.8 ± 1^{ab}	77.6 ± 2^{b}	105.5 ± 7^{b}
5 % EtOH + 20 mg/L SO_2	20 ± 0^{A}	3.84 ± 0^{B}	3.2 ± 1^{B}	3.2 ± 0^{B}	3.2 ± 0^{B}	125.2 ± 1^{a}	68.8 ± 1^{ab}	84.8 ± 5^{b}	130.4 ± 5^{a}
10 % EtOH + 20 mg/L SO_2	20 ± 0^{A}	4.8 ± 0^{BC}	3.8 ± 0^{C}	3.2 ± 0^{D}	3.2 ± 0^{D}	68.8 ± 2^{c}	93.1 ± 1^{a}	111.2 ± 1^{a}	119.0 ± 6^{ab}
12 % EtOH + 20 mg/L SO_2	20 ± 0^{A}	15.4 ± 0^{B}	14.8 ± 1^{B}	14.8 ± 1^{B}	12.5 ± 0^{C}	8.1 ± 0^{f}	7.2 ± 1^{e}	4.5 ± 1^{e}	0.1 ± 0^{e}

*The values are shown with mean ± standard deviation (SD) obtained from replicated samples (duplicate) within the same experiment. In the free SO_2 column, statistical calculations were performed on each row (differences in the sample itself over time), which are represented by capital letters. In the columns with acetaldehyde analyses, treatments at each time point are compared (differences per column at each time point) and are represented with lower-case letters. Distinct letters are significantly different according to the Tukey test ($P \leq 0.05$).

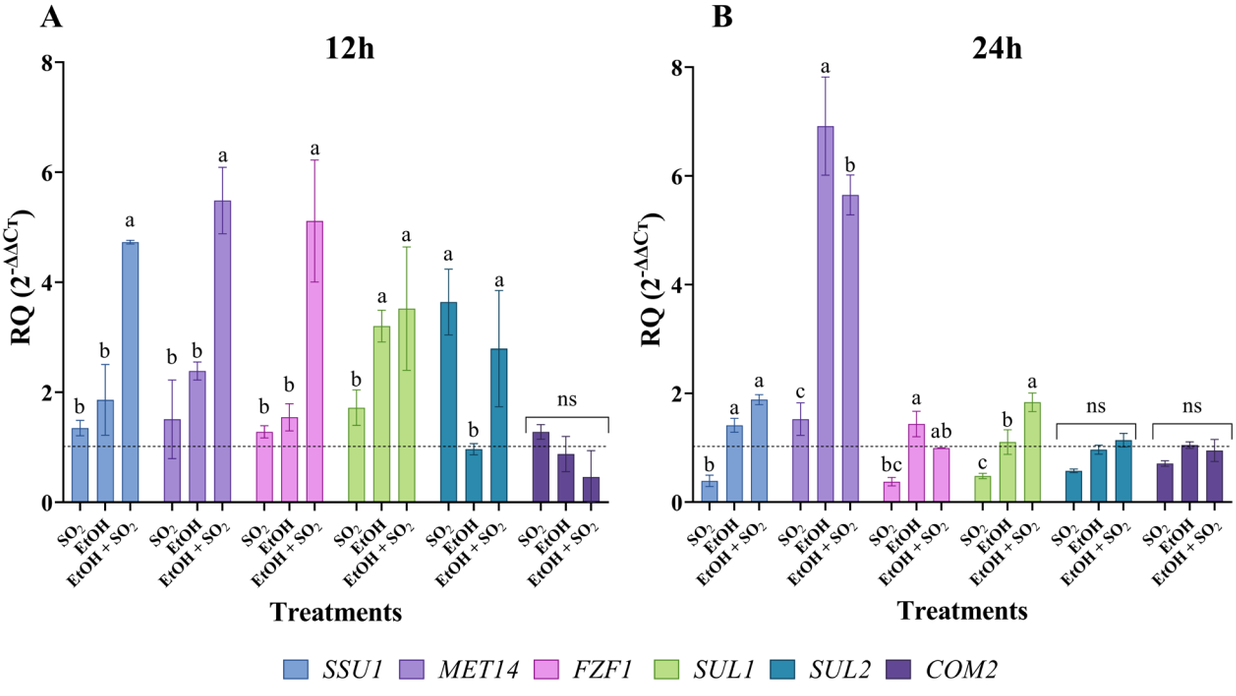

FIGURE 6. Analysis of relative expression (RQ). The dotted line represents Value 1 of the control treatment. Bars represent RQ values of each gene relative to the control treatment (dotted line). SO_2 = 20 mg/L SO_2; EtOH = 10 % EtOH; EtOH + SO_2 = 10 % EtOH + 20 mg/L SO_2. The error bars are the standard deviation of three replications of the same treatment. Different letters denote significantly different mean values by the Tukey test ($P \leq 0.05$); ns = not significant.

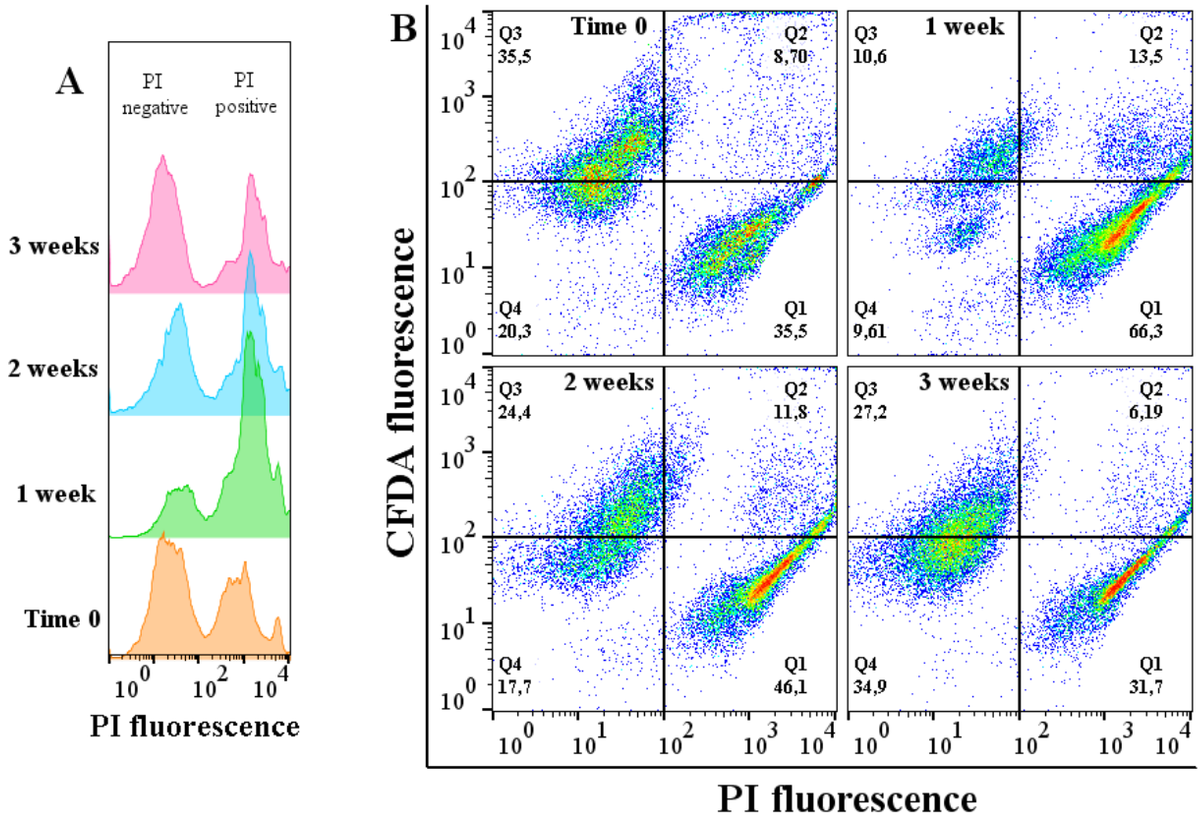

FIGURE 7. Flow cytometer analysis of the first four weeks of fermentation using the LIVE/DEAD™ FungaLight™ Yeast Viability Kit. The graph as a histogram (PI) (A). Cell concentration map (CFDA/PI) (B).

3. Vitality and viability of yeasts on an industrial scale

Figure 7 shows yeast vitality and viability during the first three weeks of the second fermentation of a sparkling wine made by the traditional method. As can be observed, just before inoculation, a lower number of cells were stained with PI, indicating a higher number of cells with an intact cell membrane (inoculum at time 0). However, one week after inoculation, there was a considerable increase in cells with damaged cell membranes (PI positive cells). Moreover, at this point, the living cells (PI negative) exhibited a lower esterase activity than the control, which reflects a reduction in cell metabolism. In the second and third weeks, the number of viable (PI negative) and overall metabolism (CFDA fluorescence) increased. In the 2nd and 3rd week, there was an increase in cells with damaged plasma membrane that prevented the entry of the dye, as well as an increase in cells containing the active esterase enzyme. During this period, free SO_2 decreased from 16.5 mg/L (time 0) to 5.5 mg/L (after 2 weeks). The behaviour of the yeasts during the industrial scale second fermentation of a real wine showed a similar effect (decrease in vitality and viability) to that in synthetic wines with similar concentrations of ethanol and free SO_2 (Figure 4).

DISCUSSION

Empirical observations in wineries report frequent problems at the start of and during the second fermentation of sparkling wines in both traditional and Charmat processes. In general, the practical solutions adopted in these cases are pre-adaptation and reinoculation with yeasts, which is costly and laborious, especially for traditional sparkling wines. Furthermore, industrial data show that these problems are associated with a considerable reduction in yeast viability. In an attempt to explain the occurrence of these problems, we carried out a series of experiments in synthetic wine to determine the effects of high concentrations of ethanol and the presence of free sulfur dioxide, and the synergism between the two, on the vitality, viability and other parameters of yeasts.

The present study shows that the synergism between ethanol and SO_2 can be considered

to be the main stress factor for yeasts at the beginning of the second fermentation (lag phase) in the production of sparkling wines. Depending on the concentrations of ethanol and SO_2, this synergism modifies cellular homeostasis, deregulates intracellular pH (loss of internal buffering capacity), increases oxidative stress, and interferes with the regulation of gene expression, which can negatively impact the vitality and viability of yeasts, slowing or even interrupting the fermentation process.

The data obtained for the synthetic wine that were supplemented with ethanol, sulfur dioxide and ethanol/sulfur dioxide (Figures 1, 2, 3 and 4), and real base wines (Figure 7) showed the synergistic effect of ethanol and sulfur dioxide on the vitality and viability of yeasts. Despite the yeasts having been adapted, the results show that whether inoculated in an environment with an ethanol concentration close to that of base wines or in an industrial base wine, yeast grows slowly but maintains high viability. This behaviour may be due to fluidisation (Huffer *et al*., 2011; Jones and Greenfield, 1987), the depolarisation of membrane potential by an increased passive proton flux and inhibition of nutrient uptake (Casey and Ingledew, 1986) and a delay in the cell cycle (Kubota *et al*., 2004). Moreover, cells grown in synthetic wine without ethanol and supplemented with 20 mg/L of SO_2 maintained a normal growth rate and high viability, as has been observed in grape must fermentations (Ferreira *et al*., 2017). Conversely, yeast inoculated in wine containing both ethanol and sulfur dioxide exhibited a rapid and strong decrease in cell viability, indicating their synergistic effect on the yeast. The low number of cells that remained viable after the initial shock slowly resumed growth, resulting in a long lag phase and slow fermentation.

The results of the experiment on synthetic musts supplemented with different concentrations of sulfur dioxide show that there was a dose-dependent longer lag phase; the yeasts started to multiply when free SO_2 concentration decayed to approximately 5 mg/L on combination with yeasts and fermentation-derived products, such as acetaldehyde (Ochando *et al*., 2020). However, in the presence of high ethanol concentrations (> 10 % v/v), yeast growth is limited (Jing *et al*., 2018), and consequently free SO_2 concentrations remain high for longer periods of time. Moreover, the presence of ethanol in real wine leads to an increase in SO_2 pKa, which, according to the expression of Henderson–Hasselbalch [free SO_2/1 + 10(pH-pKa1)] increases the molecular SO_2 fraction and consequently its antimicrobial activity.

The reduction in yeast viability during the beginning of fermentation in the presence of sulfur dioxide, and particularly sulfur dioxide and ethanol, is associated with the loss of cell membrane integrity (Figure 2), which is considered a marker of necrotic death (Wloch-Salamon and Bem, 2013). Furthermore, the cells which survived the exposure to ethanol/SO_2 showed a large increase in the intracellular concentration of ROS (Figures 2E and 3C). Experimental data (Figure 3C) indicates that ROS accumulation in ethanol/sulfur dioxide interaction depends on cell metabolism. The accumulation of ROS is one of the main determinants of apoptotic cell death (Farrugia and Balzan, 2012). Apoptosis can function as a defence and preservation mechanism of cell populations in the face of stress (viral pathogens, homeostasis change, nutrient insufficiency and other adverse conditions), ensuring that part of the cells survive to propagate their genome (Fröhlich and Madeo, 2000). When comparing both results obtained regarding ROS accumulation (Figures 2E and 3C), it is possible to observe a difference in fluorescence formation kinetics and intensity. This difference may be related to the temperature at which both experiments were conducted (15 °C Figure 2 and 20 °C figure 3) at the time of the analysis; the populations were at different theoretical stages of fermentation, since the fermentation temperature modifies metabolism and fermentation kinetics (Alexandre, 2019).

It is known that the intracellular pH of yeasts decreases in the presence of sulfur dioxide (Pilkington and Rose, 1988). In an aqueous solution, SO_2 (acid oxide-sulfur dioxide) in its molecular form (SO_2) enters cells more easily, because it has no charge, and the molecule rapidly dissociates to form bisulphite (HSO_3^-) and sulphite (SO_3^{2-}) anions (Divol *et al*., 2012). SO_2 behaves like a weak acid in aqueous environments (Waterhouse *et al*., 2016). Moreover, studies of changes to cell membrane caused by ethanol have shown that it increases passive water transport (Madeira *et al*., 2010), modifies cell membrane fluidity and decreases H^+-ATPase activity, which is responsible for maintaining intracellular pH (Aguilera *et al*., 2006). This modification to membrane structure may facilitate the diffusion of molecular SO_2 into the cytoplasm, which has no charge. The molecular SO_2 fractions found in a solution depends on the pH for a

modification of the dissociation constant to occur ($SO_2 + H_2O \leftrightarrow SO_2 \cdot H_2O$; $SO_2 \cdot H_2O \leftrightarrow HSO_3^- + H^+$; $HSO_3^- \leftrightarrow SO_3^{2-} + H^+$) (Divol *et al*., 2012; Gould and Russell, 2003; Ribéreau-Gayon *et al*., 2006; Waterhouse *et al*., 2016). The reduction of intracellular pH (loss of internal buffering capacity) may be the result of a "snowball effect", because the lower the cell's intracellular pH, the higher the percentage of the molecular form of SO_2 that would theoretically remain active within the cell, leading to the disruption of cell homeostasis and thus cell death. This hypothesis is reinforced by ethanol having a dose-dependent effect when in association with sulfur dioxide (Figure 3).

Another interesting fact is the size of yeast cells depends on their environment. In the presence of ethanol, there is a tendency for cell structure to change and for part of the cell population to become larger (it becomes swollen) (Figure 5); such an effect on the diameter of *Saccharomyces cerevisiae* cells has been described in other studies (Dinh *et al*., 2008; Kubota *et al*., 2004). However, death caused by the presence of SO_2 and ethanol decreases cell size (Figure 5). Our results indicate that there may be a relationship between cell size and loss of plasma membrane integrity (PI + cells); the percentage of inactivated cells is almost the same as that of cells that become smaller in treatments with 10 % EtOH + SO_2 20 mg/L and 12 % EtOH + SO_2 20 mg/L.

In alcoholic fermentation, the production of acetaldehyde by yeasts plays an important role when in combination with free SO_2 (Rankine and Pocock, 1969), because it decreases the antimicrobial action of SO_2 (Liu and Pilone, 2000). The excess of acetaldehyde produced by the decarboxylation of pyruvate during fermentation is secreted, instead of being used in the production of ethanol or acetic acid (Liu and Pilone, 2000). Increased acetaldehyde has been reported in the second fermentation of sparkling wines (Pozo-Bayón *et al*., 2003), and more acetaldehyde has been found to form in some yeasts (including the EC1118 strain) in the presence of exogenous SO_2 in must fermentations (Li and Mira de Orduña Heidinger, 2020; Li and Mira de Orduña, 2017). However, in the literature, no correlation between the increase in extracellular acetaldehyde and greater stress during the second fermentation of wines in the presence of EtOH and EtOH + SO_2 has been made, in contrast to the present study (Table 2). Our results point to a greater formation of acetaldehyde when ethanol and SO_2 are present together, which corroborates with the results that show an increase in stress when both molecules are present. Another important factor is the relationship between the formation of acetaldehyde by yeasts and their overcoming stress and consequently leaving the lag phase. This was clearly seen in the treatment with 10 % EtOH + 20 mg/L of SO_2 in which there was a delay in acetaldehyde production, probably due to reduced viability (Figures 3A and 4). In the treatment with 12 % EtOH + 20 mg/L of SO_2 practically no acetaldehyde was formed to combine SO_2 and overcome stress, and almost the entire yeast population was inactivated (Figures 3 and 4).

The regulation of gene expression plays an important role in an organism's development and its response to physiological and environmental changes; it can be responsible for the survival of a group of cells and its growth after a period of latency. The results obtained in terms of relative gene expression showed that, compared to the other treatments, there was an increase in the expression of several of the genes involved in sulfur sensing and metabolism in the 10 % EtOH + 20 mg/L SO_2 treatment after 12 and 24 hours (Figure 6). This agrees with the results of the other analyses that show a synergistic increase in stress in this treatment.

The *SUL1* and *SUL2* genes encode proteins located in the plasma membrane with sensory and sulphate (SO_4^{2-}) transport functions from the external environment into the cell (Kankipati *et al*., 2015). While the *SUL2* was the most expressed in treatments with SO_2 only and *SUL1* the most expressed in ethanol only, both these genes were hyper expressed in the EtOH + SO_2 treatment (Figure 6). Moreover, in the EtOH + SO_2 treatment, *FZF1* and *SSU1* are more expressed than in the other treatments. The *FZF1* gene encodes a plasma membrane protein (Fzf1p) involved in the expression of the *SSU1* sulphite efflux pump (Avram *et al*., 1999; Park and Bakalinsky, 2000). In turn, the *SSU1* gene, a marker of adaptive evolutionary advantage that is found in oenological yeast as a result of the use of sulphites in winemaking (García-Ríos *et al*., 2019; Zimmer *et al*., 2014), is positively correlated with the tolerance and detoxification mechanism of sulphite (Marullo *et al*., 2020; Zara and Nardi, 2021).

The *MET14* gene, which encodes the APS kinase enzyme involved in the sulfur assimilation pathway that reduces sulphate to sulphide (Donalies and Stahl, 2002; Noble *et al*., 2015), was hyper-expressed in the first 12 h of the EtOH + SO_2

treatment. However, after 24 h, gene expression seems to have been more influenced by the presence of ethanol only. As it is a metabolic route gene linked to the biosynthesis of sulfur amino acids, other factors - in addition to the presence of exogenous SO_2 - may influence its greater expression, such as the formation of sulphites by the yeast (Donalies and Stahl, 2002). Surprisingly, no change in expression was found for *COM2*, a gene whose product controls, directly or indirectly, the expression of more than 80 % of the genes activated by SO_2 (Lage *et al*., 2019).

In general, the increase in the expression of these genes may indicate a response to a greater amount of intracellular SO_2 caused by the synergistic effect of ethanol/sulfur dioxide, as, theoretically, more proteins related to this stress would need to be synthesised to create an efficient sulphite efflux in order to survive.

In practical terms, the data obtained in this study show that winemakers should be particularly careful when adding sulphites to base wines for the production of sparkling wines; depending on their concentrations, free sulfur dioxide in the presence of ethanol can lead to the death of a large part of yeast population, and consequently to either the non-development of the second fermentation or a slow fermentation with a long latency phase. It is worth remembering that, when added to base wines to avoid microbial instability and oxidation, sulfur dioxide will remain as free SO_2, since most of the compounds which commonly bind to sulfur dioxide are removed from the lees of the first fermentation and during the stabilisation and filtering processes. Moreover, to attain 11 to 12 % ethanol in sparkling wines, the ethanol concentration of base wines must contain more than 10 % ethanol, which - as seen in this study - interacts with sulfur dioxide, thus increasing the risk of problems in the second fermentation. Therefore, sulphites may literally be responsible for headaches in consumers (Silva *et al*., 2019), but their high concentrations in the production of sparkling wines can also be the cause of "headaches" for winemakers.

CONCLUSIONS

Depending on their concentrations, the presence of both ethanol and SO_2 in the fermentation environment causes synergism and increases stress on yeasts by modifying intracellular homeostasis, deregulating intracellular pH and increasing oxidative stress, thus leading to cell death. Moreover, ethanol/sulfur dioxide causes yeasts to hyper-express genes related to sulphite tolerance in an attempt to overcome their negative effects. A reduction in the vitality and viability of a yeast population can either prevent the second fermentation of sparkling wines from occurring at all or increase its lag phase, thus causing serious technological problems that are difficult to overcome.

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Differences in yeast behaviour during ageing of sparkling wines made with Charmat and Traditional methods

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ABSTRACT

In this study, we followed the yeast and wine behaviour during the second fermentation and subsequent lees ageing of sparkling wines produced by Traditional and Charmat methods at an industrial scale. During this period, we conducted physicochemical, microbiological, gene expression, and marker analyses of characteristics related to wine ageing. Our results show that the yeast behaviour during the fermentation is similar in both methods. However, after fermentation, there is a faster decrease in yeast vitality and viability in the Charmat method, together with an increase in the expression of autophagy-related genes (*AMS1, APE1*, and *ATG8*). We relate these factors to ageing with the continuous homogenization of the liquid practised in the Charmat method, and static ageing with the lees concentrated at the bottom of the bottle performed in the Traditional method. Despite the variation in yeast viability during ageing, there are no differences in soluble proteins, free amino nitrogen, total phenols, antioxidant activity, and colour evolution between the wines produced by the two methods, assuming few differences over the time that monitoring was conducted.

1. Introduction

There are two main methods of vinification of sparkling wines with a second fermentation (*prise de mousse*) of the dry base wine. One of these is generally called Charmat in which the refermentation takes place in an isobaric tank, and the other is generally called Traditional or Classic, in which the second fermentation takes place in sealed bottles (Butnariu, 2020; Buxaderas et al., 2022; Buxaderas and López-Tamames, 2010, 2012). Throughout this stage, yeast cells often experience stress conditions, such as relatively high ethanol concentration and the presence of SO_2 (Cisilotto et al., 2021) nutrient starvation, low pH, and temperature (Borrull et al., 2015; Martí-Raga et al., 2015), and CO_2 overpressure (Porras-Agüera et al., 2020).

In addition to ethanol and other volatile compounds, sparkling wines contain several non-volatile compounds such as proteins, peptides, polysaccharides, monosaccharides, lipids, fatty acids, phenolic compounds, among others, originated from grapes, yeasts, or produced during the fermentation process (Carrascosa et al., 2011). Some of these compounds or their precursors are released from yeast by cellular autophagy and autolysis, a phenomenon that occurs during wine ageing in the lees (Alexandre, 2019).

In oenology, both ageing on lees and the yeast autolysis process are considered an important stage in natural cell ageing or induced due interference of physical, chemical, or biological agents (Babayan and Bezrukov, 1985). The importance of this phenomenon in the wine industry resulted in several original studies related to the ageing of wines on lees and the release of cell components (Comuzzo et al., 2015; Dimopoulos et al., 2018; Gnoinski et al., 2021a, b; Gonzalez et al., 2003; Liu et al., 2016; Martínez et al., 2019, 2018; Velázquez et al., 2016). However, the phenomena that precede death and autolysis, such as autophagy and apoptosis, are still poorly elucidated under oenological conditions (Cebollero et al., 2005, 2008; Cebollero and Gonzalez, 2006a; Porras-Agüera et al., 2019).

In the Traditional method, maturation in contact with the lees (*sur lie*) can take months or years (Di Gianvito et al., 2019; Ribéreau-Gayon et al., 2021), while in the Charmat method, the ageing with the lees is normally limited to six months on less. This difference is considered to attribute more complex sensory characteristics to Traditional sparkling wines, and more fresh and fruity characteristics to Charmat wines (Di Gianvito et al., 2019; Moreno-Arribas and Polo, 2009). Many of these

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organoleptic differences have been associated with the release of yeast components during ageing with the lees by autophagic/autolytic processes (Di Gianvito et al., 2019). In this sense, some authors (Buxaderas and López-Tamames, 2012; Jackson, 2020) pointed out that Charmat sparkling wines with longer contact with the lees can release yeast compounds and acquire characteristics more like those obtained by the Traditional method.

Comparing the Traditional and Charmat methods, there are some obvious differences between the processes: (1) the volumes of base wine during foaming (*prise de mousse*) and ageing on the lees (*sur lie*), wherein one method the second fermentation occurs in a bottle (small volume), in the other, it occurs in pressure tanks (large volume); (2) the contact material with the liquid in glass (Traditional) and stainless steel (Charmat); (3) the Charmat method include the possibility of filtration and clarification, while the Traditional method just clarification (Di Gianvito et al., 2019; Ribéreau-Gayon et al., 2021; Togores, 2018). Another difference between the two methods, but little discussed, is that during the second fermentation and ageing of the wine in contact with the yeasts, in the Traditional method, lees normally remain accumulated at the bottom of the bottle, which remains in a horizontal position. The bottle is considered a small container, for this reason, there would be a greater interaction of the lees with the wine, which is considered an advantage of the Traditional method (Ribéreau-Gayon et al., 2021; Togores, 2018). Conversely, in the Charmat method, if the pressure tanks have internal stirrers, continuous stirring can be performed during the entire process (fermentation and ageing on lees), suspending the lees, and leaving the wine always homogenous (Jackson, 2020). The continuous stirring makes the contact of the lees with the liquid greater than in the Traditional method. This technological option could influence several factors related to yeast metabolism and the release of compounds, but we did not find studies that specifically address this process in the elaboration of sparkling wines.

Although these two methods of making sparkling wine are widely used, few studies have been conducted comparing the behaviour of the yeasts and the characteristics of the wines in the two methods. The particularities of each method could modify the life cycle of the yeasts and consequently influence the composition and concentration of biomolecules released by the yeasts in the sparkling wine. For this reason, we tested on an industrial scale whether, from the same base wine, the method used in the second fermentation interferes with the behaviour of the yeasts and the composition of the sparkling wine during fermentation and subsequent ageing on lees.

2. Materials and methods

2.1. Base wine

The base wine used in the second fermentation in both methods was a blend (*assemblage*) of wines from the Chardonnay (36%), Riesling Italic (30%), and Pinot Noir (34%) grape varieties (vinified in white). Before the second fermentation, the wine underwent tartaric stabilisation for 20 days at −2±1 °C, followed by filtration (Bucher Vaslin tangential filter - Flavy FX 06, France). As an analytical profile, the wine presented: 1 g/L of reducing sugars; 11.2% alcohol (v/v); 6 g/L of total acidity expressed as tartaric acid; 3.27 pH; 0.28 g/L of volatile acidity; 14 mg/L of free sulfur dioxide, and 80 mg/L of total sulfur dioxide. No readily assimilable nitrogen was added to the base wine.

2.2. Inoculum for second fermentation

The yeast of the species *Saccharomyces cerevisiae* strain LALVIN® EC-1118 (Lallemand, Canada) in active dry form was used. This yeast strain is recommended and conventionally used in the second fermentation of sparkling wines and its genome has already been sequenced (Novo et al., 2009), a factor that facilitated the molecular analyses. After hydration (manufacturer's protocol), adaptation to the alcoholic medium was done gradually (1st day 25% wine and 75% water; 2nd day 50% each; 3rd 75% wine 25% water and from 4th day only wine), along with population multiplication, and volume increase. Sugar concentration was maintained near 15 g/L, and readily assimilable nitrogen was added at a rate of 0.75 g for each 15 g of added sugar (organic and inorganic nutrients with a percentage of approximately 35% and 65% respectively). This process was carried out in a specific tank with a temperature control system (12 °C), homogenization (20 min of homogenization followed by 80 min without homogenization), and aeration (3 min compressed air injection followed by 180 min without injection). The increase in volume and multiplication of the inoculum (*pied de cuve*) was performed according to the company's protocol. The percentage of ethanol from the inoculum at the time of inoculation was 13.3% v/v and the total yeast population was 3.5×10^7 cells/mL with viability of approximately 70%.

2.3. Second fermentation and ageing of sparkling wines

The second fermentation of the sparkling wines in the Charmat and Traditional methods were carried out at the Chandon Brazil winery (Garibaldi, RS - Brazil) on an industrial scale (real oenological conditions). In a vertical pressure tank (50,000 L with an internal stirrer of the rotating helix type), approximately 22 g/L of sucrose and the previously acclimatised inoculum (4% v/v of the total volume) were added to the wine. After the mixture was homogeneous, a cell count and viability analysis were performed. A population of approximately 1.4×10^6 cells/mL was estimated with viability close to 70% (analysis performed with an optical microscope, Neubauer chamber, and methylene blue dye). A portion of the wine was then bottled (Traditional method) in specific sparkling wine bottles with a volume of 750 ml. The closure was made with a plastic bidule and a metal crown cap model TOP+ (PE.DI, Italy). The remaining wine stayed in the pressure tank to perform the second fermentation by the Charmat method. The agitator (internal propeller) of the pressure tank remained in operation (60 rpm) throughout the fermentation and ageing period, allowing the liquid to always remain homogeneous with the lees in suspension. The bottles remained immobile in a horizontal position during the whole period, until the collection of the samples. The second fermentation in both methods was conducted at a controlled temperature of 12 ± 1 °C. It was considered the end of fermentation when the amount of reducing sugars was below 3 g/L. The fermentation time was approximately 11 weeks (77 days). No clarifiers were used during foaming in either method so as not to influence flow cytometry analysis and further comparisons.

After the fermentation and during the maturation period on the lees, the tank (Charmat method) had its temperature reduced to 6 °C (the method used by the company). The bottles (Traditional method) were partly kept in a controlled environment at a temperature of 6 °C (positive control) and partly at a temperature of 10 °C (close to that commonly used in sparkling wine maturation cellars).

2.4. Sample collections

For the analyses during the second fermentation and subsequent ageing, 3 bottles and a tank sample volume of approximately 2 L were collected at each time. At each interval physicochemical analyses and cell viability monitoring by flow cytometry, microscopy and plating were performed. Moreover, wine and yeast cell samples were frozen at −20 °C and −80 °C, respectively, for further biochemical and gene expression analysis. The total follow-up time of the experiment was 96 weeks.

2.5. Confirmation of the effect of continuous homogenization on viability loss

A second trial with another sparkling wine was carried out to verify the phenomenon of accelerated yeast death by continuous

homogenization. After the end of the second fermentation of a sparkling wine made by the Charmat method, bottles were filled in such a way that ageing on the lees also occurred with the bottles in a horizontal and immobile position. In the tank, the agitator (internal propeller) remained always on, and the liquid was homogeneous. In this case, both the tank and the bottles remained during all this period at a temperature of 6 °C. The viability follow-up lasted 20 weeks.

2.6. Physicochemical analyses and microscopic yeast viability assay

The pressure inside the bottles was measured using an aphrometer, and in the tank using a manometer. Both were expressed in atmospheric pressure (atm) at 20 °C. The wine density was determined by direct reading with a specific gravity hydrometer (0.900/1.000 scale) and was expressed in g/mL. Reducing sugars (expressed in g/L) were quantified by the modified Lane-Eynon procedure (Zoecklein et al., 1990). Ethanol was determined by distillation in Super DEE (Gibertini, Italy), and the measurement of distillate density at 20 °C with a hydrometer alcohol meter and expressed as a volume-volume percentage (% v/v). The pH was determined with a Thermo Electron Orion Model 310 pH meter (EUA), according to the manufacturer's instructions. Total acidity (TA) was measured by titration with 0.1 N sodium hydroxide solution using bromothymol blue as an indicator, and the results were expressed in g/L tartaric acid (OIV, 2015). Quantification of volatile acids determined by steam distillation in Super DEE (Gibertini), followed by titration, and expressed as g/L of acetic acid (OIV, 2009). Free and total SO_2 were evaluated Ripper titration method (Zoecklein et al., 1999), and expressed in mg/L of SO_2.

The yeast viability evaluation (exclusion tests) was performed by cell counting on a light microscope with a 400× total magnification Zeiss West 403,036 (Thuringia, Germany) in a haemocytometer Neubauer chamber. Viability was determined by staining (1/1 v/v) with a 0.1% methylene blue solution plus 2% sodium citrate (Gilliland, 1959). The number of unstained cells divided by the total number of cells X 100 represents the percentage (%) of viable cells. For yeast survival plating, suspensions were serially diluted, seeded in solid YPD agar (1% yeast extract, 2% peptone, 2% glucose, and 2% agar) and after incubation (28 °C - 24 h) the colony forming units (CFU) were counted. The viability curve was calculated as the percentage of CFU with respect to CFU at the stationary phase, referred to as 100% survival.

2.7. Cell vitality and cell viability with flow cytometry analysis

To perform the cellular analyses by flow cytometry, samples were centrifuged to separate the cells (4629×*g* for 5 min), and after yeasts were washed with phosphate-buffered saline pH 7.2 (PBS). Flow cytometry analyses were performed in a FACSCalibur flow cytometer (Becton-Dickinson, USA) equipped with an argon-ion laser emitting at 488 nm. The flow cytometer data of 20,000 cells were acquired using CellQuest Pro software (BD Bioscience) and data analysis was carried out using FlowJo v.10 software (TreeStar, Inc, USA).

The cellular vitality and cellular viability (cell membrane integrity) were determined using the fluorescent dye LIVE/DEAD™ FungaLight™ Yeast Viability Kit (Thermo Fisher Scientific, EUA), which includes 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA), which is cleaved by nonspecific esterases resulting in a fluorescent product, and propidium iodide (PI), that penetrates only in membrane damaged cells. The staining and flow cytometry analyses were performed according to the manufacturer's recommendations. All samples were incubated for 30 min in the dark before analysis.

2.8. Gene expression (qRT-PCR)

The samples for analysis were centrifuged to separate the cells (4629×*g* for 5 min) and then the pellet of cells was frozen in a freezer at −80 °C, remaining until the moment of RNA extraction. RNA extraction was performed according to a method specific for *Saccharomyces cerevisiae* (Shedlovskiy et al., 2017). The extracted RNA was treated with DNase I (Thermo Fisher Scientific) following the manufacturer's protocol. The absence of contaminant genomic DNA in the RNA was checked before cDNA synthesis using RNA as a template for a PCR assay. The RNA was reversed-transcribed into cDNA with the *M*-MLV Reverse Transcriptase (Thermo Fisher Scientific) following the manufacturer's protocol. The primers used in qRT-PCR are shown in Table 1. Their sequences were obtained from published studies and by using the Primer designing tool system on the NCBI website (Primer-BLAST). Primers were purchased from Thermo Fisher Scientific.

Real-time PCR was performed in Applied Biosystems StepOne qRT-PCR (Thermo Fisher Scientific) using SYBR Green as a fluorophore. Reactions were carried out in 20 μL of mix containing 10 μL MasterMix Real-Time PCR - SYBR Green/Rox 2× dye (Ludwig Biotecnologia Ltda, Brazil), 2.0 μL of primer mix (200 nM final concentration), and 8 μL of cDNA (diluted 1/15). The thermocycling program consisted of one hold at 95 °C for 2 min, followed by 40 cycles of 15 s at 95 °C, and 30 s at 60 °C. After the cycles, melting-curves data were collected to verify PCR specificity and contamination. Moreover, in an initial experiment, three potential housekeeping genes were evaluated: *ACT1*, *TEF1*, and *IPP1*. As all of them gave similar Cq values for the different samples, *ACT1* was selected for further analysis. All reactions were run in duplicate and normalized to the expression of *ACT1*. The relative mRNA abundance was calculated by the comparative quantification cycle (Cq) method (Livak and Schmittgen, 2001), where the Cq is the PCR cycle number at which the sample fluorescence signal passes a fixed threshold line and is reported as $2^{-\Delta Cq\ (mRNA\ target\ -\ mRNA\ ACT1)}$.

2.9. Quantifications of proteins, free amino nitrogen , total phenols, antioxidant activity, and wine colour evolution

The sparkling wine soluble proteins content was determined according to the Bradford method modified for microplates (Brogdon and Dickinson, 1983) using the VWR Life Science Bradford Method Protein Assay Kit (USA) following the manufacture instructions, with a bovine serum albumin standard curve ($R^2 = 0{,}989$). The absorbance (Abs) was determined at 595 nm with a microplate absorbance reader (Biochrom Asys Expert Plus, UK). The results were expressed as mg/L.

The analysis of free amino nitrogen (FAN) was determined with the ninhydrin method and microplate absorbance reader (Abs 575 nm) (Abernathy et al., 2009). A standard curve was performed with the amino acid glycine ($R^2 = 0{,}998$). The results were expressed as mg/L of FAN (glycine equivalent).

Total phenolic compounds were determined by the Folin-Ciocalteu colorimetric method according to a microscale protocol (Waterhouse, 2002). A standard curve with gallic acid ($R^2 = 0{,}999$) was performed. The measure of the sample was performed absorbance at 765 nm with a spectrophotometer (Biochrom Libra S12, UK). Results were expressed as mg/L of gallic acid equivalent.

The DPPH (2,2-Diphenyl-1-picrylhydrazyl) reagent was used to determine the antioxidant activity (scavenging activity). The analysis was performed in a microplate absorbance reader (Abs 515 nm) (Bobo-García et al., 2015). The inhibition percentage of DPPH at the steady-state was determined using the equation [% DPPH inhibition = (absorbance blank - absorbance sample)/absorbance blank x 100] (Carmona-Jiménez et al., 2014).

The yellow colour (Abs 420 nm) and tint (Abs 420 nm/Abs 520 nm) of wines was determined by absorbance using a Pró-tools UV–1600 Spectrophotometer (Shanghai Mapada Instruments, China) and expressed as optical density units (Ribéreau-Gayon et al., 2021).

2.10. Statistical analysis

Quantitative data were analysed by One-way ANOVA followed by means comparison by the Tukey's test ($P \leq 0.05$) or two-tailed unpaired

Table 1
Genes and primers used in qRT-PCR.

Gene	Primer F (5' - 3')	Primer R (5' - 3')	Size (bp)	Marker	Reference
ACT1	TCGCCTTGGACTTCGAACAA	CAAAGCTTCTGGGGCTCTGA	128	Housekeeping gene	Cisilotto et al. (2021)
IPP1	AGCCAGTTTCTGCCTTCCACGA	TGGTGATTTCTAACTTGGCGTTGGT	111	Housekeeping gene	Cisilotto et al. (2021)
TEF1	GGTTACTCTCCAGTTTTGGATTGTC	ACGAACTTGACCAAAGCAGC	149	Housekeeping gene	Nardi et al. (2010a)
AMS1	ACCAAAGGCTCCGGATGCGC	TGAGCGAGCTTAACAGTGTCGCT	105	Autophagy	This paper
APE1	AGCACGCCGCAATGGAGACA	TGCTGTGCATGGACAACTGTGCA	143	Autophagy	Zampieri et al. (2014)
VPS34	GTGCTGGCTATTGTGTTATTACATACATC	ATGGCGGAAAAGGTTTGGGG	141	Autophagy	Nardi et al. (2010b)
ATG8	GAAGGCGGAGTCGGAGAGG	GGCAGACATCAACGCCGC	238	Autophagy	Mendes-Ferreira et al. (2010)
YCA1	GGTCCTCCCCCTATGGCTTA	TGGGAACCCTGACCAAATCG	385	Apoptosis	Gowsalya et al. (2019)
AIF1	GTGTTTGGTGTGTCTGTGGC	AAAGCTGGCAGCCGTATCTT	207	Apoptosis	This paper
NUC1	TTCGATTGTTTGATCGCCGC	TATTGCTGTCGCGGCATTTG	213	Apoptosis	This paper
SOD1	TGTCGGTGACATGGGTAACG	AACGACGCTTCTGCCTACAA	115	Oxidative stress	This paper
GLR1	CGACCGGTGGAAAGGCTATT	CAGCGCCAACAACAACAACT	117	Glutathione redox state	This paper
PGK1	GCTGCTTTGCCAACCATCAA	AATAACGGAACCTGGGGCAG	225	glycolysis and gluconeogenesis	This paper
ENO2	GGTAACCCAACCGTCGAAGT	CGACGGCCTTTTGGTCCTTA	226	glycolysis and gluconeogenesis	This paper
GNP1	GGTTCAAAGGTGTCGTTGCC	GAGAGGAGACAGCGATGACG	264	Amino acid permease	This paper
GAP1	TACTGCGCAACGTCTCCAAT	TACCTTCGTTGCTTGCCTCA	180	Amino acid permease	This paper
PDC1	AGCATCCTTGACCAAAGCCA	TGAACACGTCGGTGTTTTGC	364	Alcoholic fermentation	This paper

t-test ($P \leq 0.05$).

3. Results

Despite the differences in fermentation between the methods and the fact that in the Charmat method the liquid is in constant homogenization while in the Traditional method the bottles remain immobile, the duration of the second fermentation was very similar (Table 2). During the second fermentation, there were no significant differences in any of the physicochemical parameters analysed, like reducing sugars, ethanol, total and volatile acidity (Table 2), and the fermentative kinetics were very similar. However, at the end of the fermentation (11 weeks), significant differences in yeast viability were observed between the two methods (Table 2).

After 9–11 weeks, when reducing sugars attained <3 g/L in both Traditional and Charmat methods, a change in the behaviour of the yeast populations occurred, with a more intense drop in vitality and viability in the Charmat method (Fig. 1). Although from week 11 the temperature of the pressure tank was lowered to 6 °C (a method used by the company), the drop in viability and vitality of the population in the Charmat method continued more intense, with almost the double that of the Traditional method (Fig. 1-A, B and C).

To confirm the effect of continuous homogenization on viability loss an experiment conducted under continuous stirring and static ageing, both at 6 °C, we observed a drastic reduction of yeast viability in the homogenized treatment (less than 5% viability after 4 weeks from the end of fermentation), while in the treatment with the lees at the bottom of the bottle (Traditional method) yeast retained 20% viability after 20 weeks (supplementary material).

Fig. 2 shows the differences in yeast vitality and viability throughout Traditional sparkling wine ageing with the lees at 6 °C and 10 °C. As can be observed, the lower the temperature, the higher the lifespan and longevity of the yeast population. At the higher temperature (10 °C) yeast viability dropped to approximately 1% after 26 weeks of ageing, while at 6 °C yeast retained 23% viability.

In the analysis conducted to quantify the expression of genes related to metabolism, stress, and cell death (Fig. 3), the results show that in the Charmat method there was a precocious increase in the amount of mRNA related to autophagy and oxidative stress. In the Charmat method, autophagy-related genes, such as *AMS1* gene (involved in free

Table 2
Analysis of sparkling wines during the second fermentation in the two methods of sparkling wine production. Viability◆ = % methylene blue negative. Data are presented as means ± standard deviation. Single comparisons were performed with two-tailed unpaired t-tests between treatments at each time and it was found that there are no statistical differences at the level tested (α = 0.05) except for viability at the end of fermentation (11 weeks), where * indicates significant differences P < 0.05, unpaired *t*-test.

Analysis	Time 0	2 weeks		3 weeks		5 weeks		7 weeks		9 weeks		11 weeks	
	Start	Charmat	Trad.	Charmat	Trad.	Charmat	Trad.	Charmat	Trad.	Charmat	Trad.	Charmat	Trad.
Pressure (atm at 20 °C)	0	0.9 ± 0	1.2 ± 0.2	2.0 ± 0	2.2 ± 0	4.0 ± 0	4.4 ± 0.3	5.6 ± 0	5.9 ± 0	6.4 ± 0	6.5 ± 0	6.75 ± 0	6.75 ± 0
Density (g/mL)	0.998 ± 0	0.997 ± 0	0.996 ± 0	0.995 ± 0	0.995 ± 0	0.992 ± 0	0.993 ± 0	0.991 ± 0	0.990 ± 0	0.989 ± 0	0.989 ± 0	0.989 ± 0	0.989 ± 0
Reducing sugars (g/L)	22.3 ± 0	19.1 ± 0	19.0 ± 0.5	15.9 ± 0	16.0 ± 0.5	9.5 ± 0.1	9.2 ± 0.1	4.7 ± 0.1	4.3 ± 0.3	3.3 ± 0	2.9 ± 0.1	2.1 ± 0	2.3 ± 0
Ethanol (% v/v.)	11.2 ± 0	11.4 ± 0	11.3 ± 0	11.6 ± 0	11.7 ± 0	12.0 ± 0	12.0 ± 0.1	12.3 ± 0.1	12.3 ± 0	12.4 ± 0	12.4 ± 0	12.5 ± 0	12.4 ± 0.1
pH index	3.27 ± 0	3.27 ± 0	3.28 ± 0	3.30 ± 0	3.30 ± 0	3.26 ± 0	3.28 ± 0	3.29 ± 0	3.30 ± 0	3.3 ± 0	3.3 ± 0	3.26 ± 0	3.28 ± 0
Total acidity (g/L)	6.0 ± 0	6.0 ± 0	6.0 ± 0	6.0 ± 0	6.0 ± 0	6.0 ± 0	6.0 ± 0	6.0 ± 0	6.0 ± 0	6.0 ± 0	6.0 ± 0	6.0 ± 0	6.0 ± 0
Volatile acidity (g/L)	0.28 ± 0	0.29 ± 0	0.30 ± 0	0.31 ± 0	0.30 ± 0	0.30 ± 0	0.28 ± 0.01	0.30 ± 0.01	0.30 ± 0.01	0.28 ± 0.01	0.30 ± 0.01	0.31 ± 0	0.31 ± 0.01
SO_2 free (mg/L)	14.1 ± 0.1	6.1 ± 0.2	6.3 ± 0.3	5.0 ± 0.2	5.5 ± 0.6	6.4 ± 0.3	6.0 ± 0.1	6.6 ± 0.2	6.3 ± 0.3	5.8 ± 0.3	5.5 ± 0.2	5.4 ± 0.3	5.5 ± 0.5
SO_2 total (mg/L)	79.5 ± 0.7	72.0 ± 0.3	73.0 ± 0.8	69.1 ± 0.5	70.1 ± 1.5	72.0 ± 0.8	72.5 ± 0.4	68.7 ± 1.2	69.3 ± 0.7	69.0 ± 1.0	70.5 ± 1.5	69.5 ± 0.5	70.5 ± 1.5
Yeast viability◆	71.3 ± 3	60 ± 1,5	60.8 ± 1,5	64.5 ± 1,8	66.7 ± 2,3	59.9 ± 1,4	63.5 ± 4,3	63.7 ± 3	63.2 ± 0,7	55.3 ± 3,9	53.1 ± 1,4	38.5 ± 3*	48.2 ± 1*

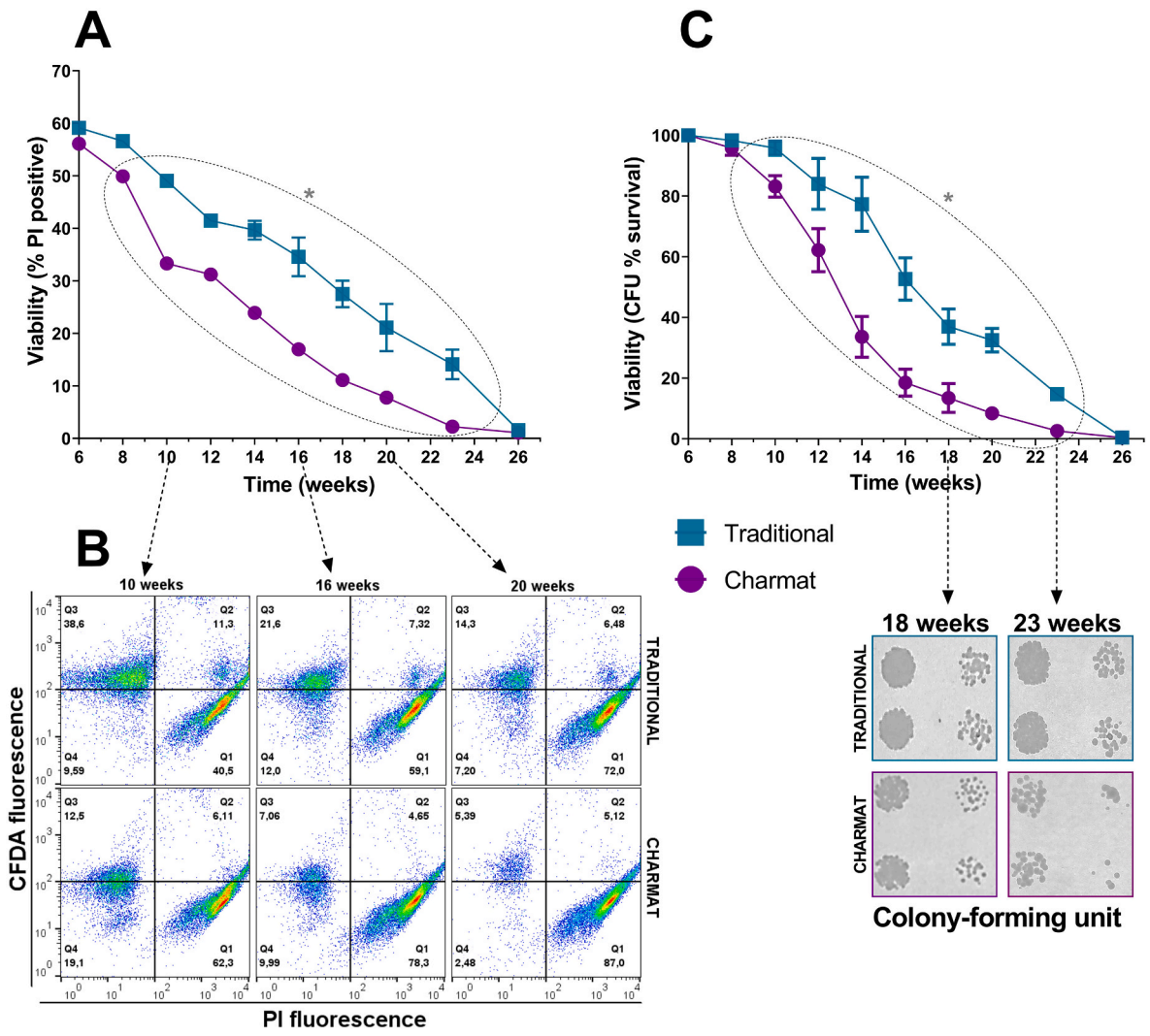

Fig. 1. Viability of yeasts during the ageing with lees. A = analysis performed with flow cytometry (PI = Propidium Iodide). B = Flow cytometry dot plots (CFDA = vitality/PI = viability); C= CFU (colony-forming unit) and Petri dish photograph (photography taken 48 h after plating). Circle with * indicates significant differences $P < 0.05$, unpaired *t*-test.

oligosaccharide degradation, upregulated under glucose starvation) (Umekawa et al., 2016; Yoshihisa and Anraku, 1989), *APE1* gene (vacuolar aminopeptidase often used as a marker protein in studies of autophagy) (Cueva et al., 1989; Suzuki et al., 2002), had a considerable increase, and, in particular, the *ATG8* gene (role in membrane fusion during autophagosome formation) (Xie et al., 2008), had a significant increase (more than 1000 times its concentration in the time "10 weeks".). This higher amount of mRNA of genes used as markers of autophagy and oxidative stress in the 10 weeks may be related to the early drop of viability in the Charmat method. In fact, in the viability follow-up analyses performed (Fig. 1-A), an accentuated drop starts in this same period. Regarding the genes used as markers of death by apoptosis, there were few changes in the two methods in the analysed periods, suggesting that soon after the second fermentation this type of programmed death mechanism does not have much influence on the decrease of viability. The genes used as markers of the glycolytic and fermentative pathway showed few differences in the two methods and along the ageing period, remain stable during this follow-up period.

In the monitoring analyses throughout the follow-up period (96 weeks), the concentration of soluble proteins, free amino nitrogen, antioxidant activity markers, and colour evolution (Fig. 4), no considerable differences were observed between the methods. Even the variations that occurred over time in the two methods are similar, with equal tendency to increase and decrease over time. Only in the yellow colour marker (Abs 420 nm), there seems to be an increment in the Traditional method in the last weeks of monitoring. These analyses were also performed on sparkling wines made by the Traditional method aged at 6 °C at 14, 18, 26, and 96 weeks (data not shown), but the values did not differ statistically from those of the Traditional method aged at 10 °C, indicating that this range of temperature did not significantly influence in these parameters.

4. Discussion

Chemical parameters (ethanol, reducing sugars, total and volatile acidity) determined during the second fermentation (*prise de mousse*) by the Traditional and Charmat methods did not differ statistically, indicating that, independent of the differences (volume and homogenization) the fermentations were similar. Moreover, although with a reduction over time, cell viability was very similar in both fermentation systems during the first weeks, but drastically dropped down after 11 weeks in the Charmat method compared with the Traditional method. This difference occurred when the concentration of sugar was <3 g/L, indicting sugar starvation as a potential cause of yeast cell death during sparkling wines ageing.

If Traditional and Charmat wines are so similar, why did yeast

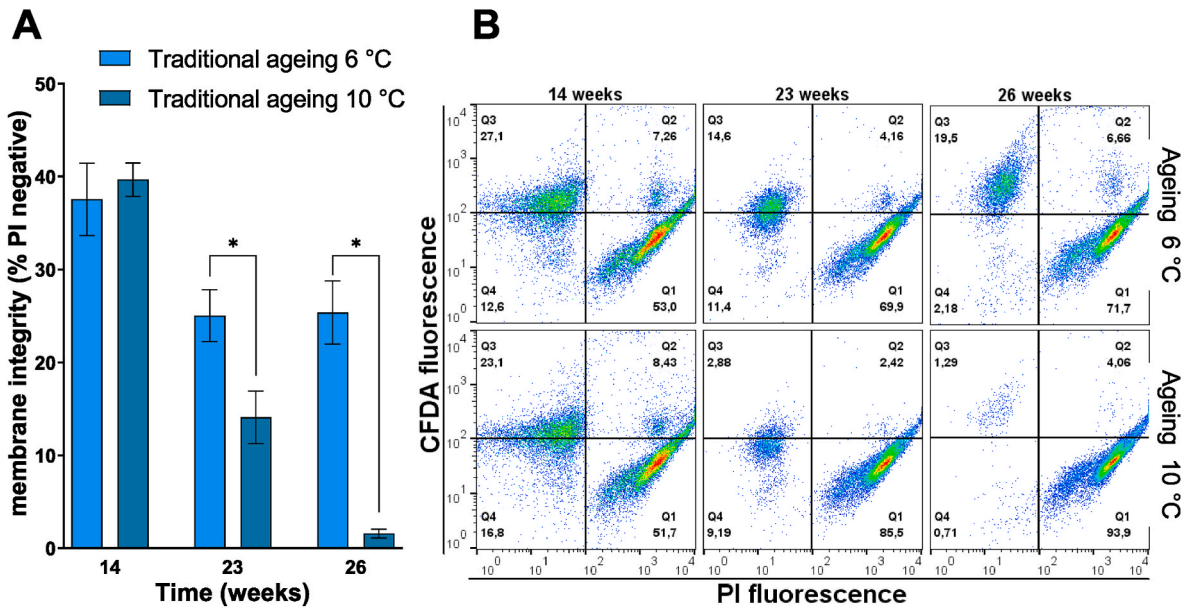

Fig. 2. Yeast ageing of sparkling wines made using the Traditional method at a temperature of 10 and 6 °C. A = * indicates significant differences $P < 0.05$, unpaired *t*-test. B = Flow cytometry dot plots (CFDA = vitality/PI = viability).

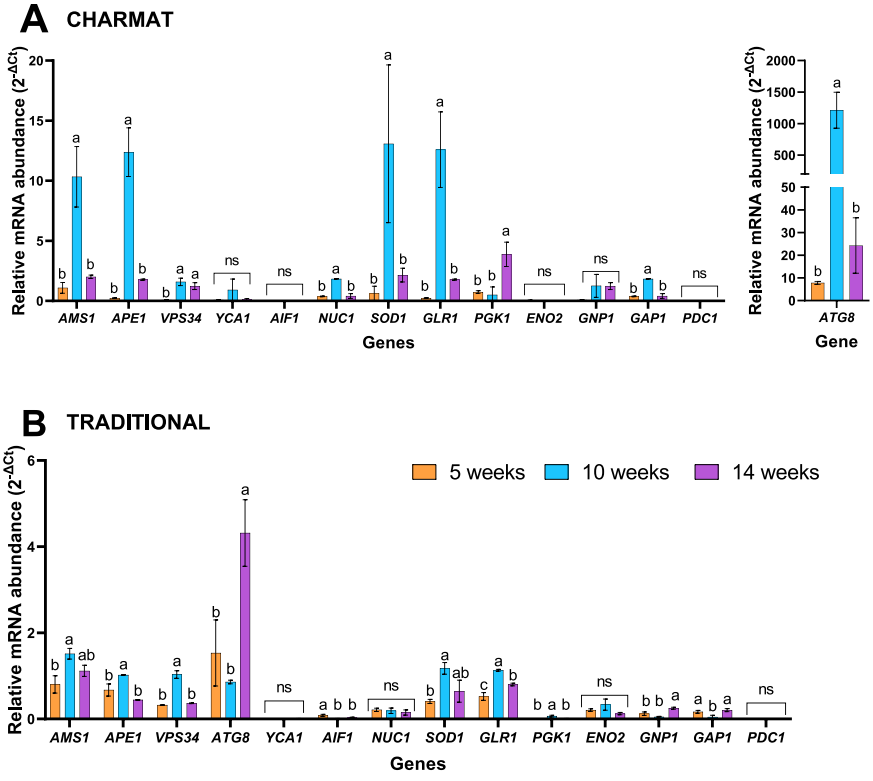

Fig. 3. Relative mRNA abundance in the final of second fermentation and initiation of ageing. For the comparison, the *ACT1* gene was used as housekeeping. Different letters indicate statistical differences between the weeks using One-way ANOVA followed by Tukey's test ($P < 0.05$). Ns = not significant. Genes related to autophagy processes = *AMS1, APE1, VPS34 ATG8*; Genes related to apoptotic processes = *YCA1, AIF1, NUC1*; Genes related to oxyreductive processes = *SOD1, GLR1*; Genes related to glycolysis and glycogenolysis processes = *PGK1, ENO2;* genes related to amino acid permease = *GNP1, GAP1;* Gene related to alcoholic fermentation = *PDC1*.

viability dropdown more intensively in the Charmat method? In this sense, we hypothesize that the main difference is associated whit the homogenization (continuous agitation) used in the Charmat method in which yeast cells are maintained in a "planktonic" system, and the static fermentation adopted in the Traditional method, in which cells are deposited in the bottom of the bottles, simulating a "benthic" system with high cell-cell contact.

This hypothesis was supported by a precocious and higher expression of macroautophagy related genes (*ATG8* and *VPS34*) and genes responsible for the most important enzymes of the macrophagosomes (*AMS 1* – mannosidase, and *APE1* – aminopeptidase) during ageing in the continuous homogenization system adopted in the Charmat method, compared with the Traditional method. Moreover, the gene expression data showed that apoptotic related genes (*YCA1, AIF1A,* and *NUC1*) are not hyper-expressed during ageing in the two systems, indicating that programmed cell death is not an important cell death pathway in the autophagic/autolytic process along sparkling wines ageing on their lees.

Autophagy, particularly macroautophagy, is considered a "pro-life" process that allows the degradation and recycling of cellular components under starvation conditions to support cell metabolism, survival,

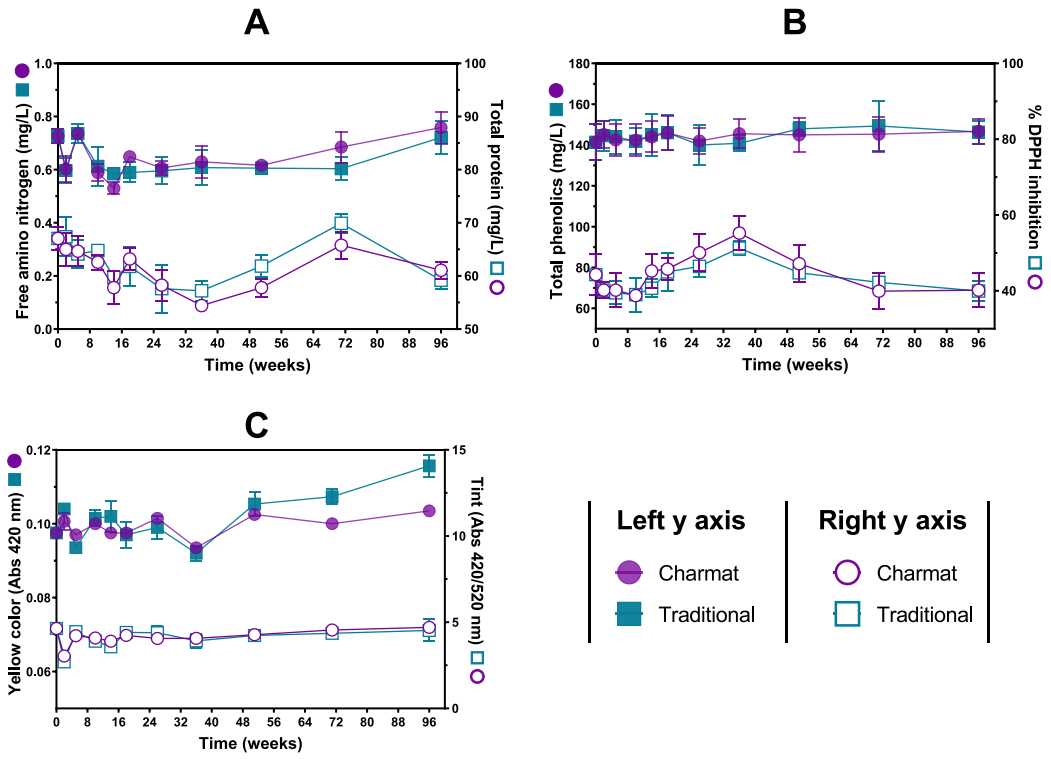

Fig. 4. Quantification and evolution over time of soluble proteins, free amino nitrogen, phenols, antioxidant activity, and colour evolution. Time 0 refers to the sample taken after inoculation. A = Free amino nitrogen and total proteins; B = Total phenolic compounds and antioxidant activity; C = Abs 420 nm representing yellow colour and tint (Abs 420/520). Free amino nitrogen is expressed as glycine equivalent; total phenolic is expressed as gallic acid equivalent. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and adaptation (Reggiori and Klionsky, 2013; Tyler and Johnson, 2018). Under starvation, yeast cells turn on the macroautophagy recycling cell proteins, lipids, wall, mitochondria, etc. However, current knowledge suggests that in drastic conditions, once the sources of nutrients available in the medium and the options for recycling the internal organelles run out, the maintenance of life becomes untenable, and it may be directly linked to the process of cell death (Liu and Levine, 2015; Noguchi et al., 2020).

During the autophagic system cell degradation products are released into the medium and can be used by other cells. If cells are close together, like in the lees deposited in the bottom of sparkling wine bottles, neighbour cells in a "benthonic" system can efficiently incorporate the components of dead cells and maintain their viability. Conversely, in a homogenized process, like the Charmat method, cell components released by autophagy/autolysis are dispersed in the medium and are not readily available by living cells. This hypothesis supports whit the results (Llaubères et al., 1987), which showed a higher release of polysaccharides from *S. cerevisiae* when the yeasts lees were stirred (*bâtonnage*) during the barrel ageing of white wines.

Several works showed that higher ageing temperature decreases the lifespan of *Saccharomyces cerevisiae* (Alexandre, 2019; Gonzalez et al., 2008; Molon and Zadrag-Tecza, 2016; Orozco et al., 2012; Tudela et al., 2012). These results were confirmed by the present study (Fig. 2). However, as can be seen in Fig. 1, the effect of continuous homogenization has more influence on the decrease of viability than the temperature during the ageing of sparkling wines. Although the Charmat method was kept at a lower temperature during ageing (6 °C), the decrease in viability was greater than in the Traditional method ageing at 10 °C. As our experiments were performed under industrial conditions, respecting the production workflow of the winery, we did not have the opportunity to test the effect of agitation at a higher temperature. However, in an additional experiment conducted under continuous stirring and static ageing, both at 6 °C (supplementary material), we observed again a drastic reduction of yeast viability in the homogenized treatment, indicating that in fact, homogenization stimulates the autophagic/autolytic process during ageing.

It is important to emphasize that the autophagic process of yeast cells is considered to play an important role in sparkling wines, contributing to the complexity, softness, and smoothness of aged wines (Cebollero et al., 2005; Cebollero and Gonzalez, 2006b). These characteristics have been associated with the release of peptides, mannoproteins, glucans, and other yeast cell components (Alexandre, 2011, 2019; Pons-Mercadé et al., 2021). In this sense, several studies focused on methods to accelerate the autophagic/autolytic process such as the use of: (1) yeast mutants (Gonzalez et al., 2003; Nunez et al., 2005); (2) microwave and/or ultrasound with the addition of β-glucanase enzymes (Gnoinski et al., 2021a, b); (3) pulsed electric field treatments (Dimopoulos et al., 2018; Martínez et al., 2016, 2018, 2019), among others strategies (Comuzzo et al., 2017; la Gatta et al., 2016; Velázquez et al., 2016). However, although demonstrating efficiency, these alternatives do not yet have a major impact on the wine industry. In this context, a better understanding of the mechanisms involved in yeast autophagy/autolysis may help to maximize and control yeast contribution in sparkling wines production.

Despite the observed differences in cell viability and gene expression upon ageing of the sparkling wines produced by the Traditional and Charmat methods, we observed almost no clear differences in amino free nitrogen, total protein, total phenolic compounds, antioxidant activity, or colour along with the ageing of the wines produced by the two methods. These results indicate that the differences between the methods are subtle. Regarding the concentrations of compounds such as free amino nitrogen and total protein, the two methods showed close values and similar behaviour over time, with no significant differences. Moreover, as expected, the antioxidant activity and total phenols analyses did not vary differences between methods. Equivalent results comparing winemaking methods have already been presented in a study

comparing winemaking methods (Caliari et al., 2015). Significant differences in antioxidant activity are mainly influenced by grape phenolic composition and the extraction of these compounds during vinification (Villaño et al., 2006). However, as with the free amino nitrogen and total protein analyses, the use of more precise methods may highlight differences between the methods, as has been reported (Stefenon et al., 2014).

In the analyses of yellow colour evolution and tint (oxidation markers), there are no differences between the methods despite the ageing temperature being different. However, in the long term, the type of bottle closure can probably have much more influence than the method of elaboration (Villedey et al., 2019). The large volume of wine in the pressure tank used in the Charmat method, internal temperature control, and the possibility of regulating the internal gases in the tank is perhaps an advantage in terms of the longevity of the product.

In a practical oenological field, the continuous agitation at the end of the second fermentation of sparkling wines could accelerate the autolysis processes due to the early death of the yeasts. Accelerated autolysis is highly desirable as it can reduce production times and costs. The continuous homogenization of sparkling wine after fermentation proved to be an efficient way to increase the cell death rate and a maybe oenological option to improve and speed up this process.

In general, regarding the elaboration methods, we found interesting differences, mainly in the increase in the speed of loss of vitality and viability of the yeasts after fermentation due to the internal agitation of the liquid in the pressure tank, together with an earlier and higher expression of autophagic related genes. This fact can be better studied, disseminated, and exploited technologically to understand more deeply in an applied way, which specific characteristics this can cause in sparkling wines in the short, medium, and long term.

5. Conclusions

During the ageing of sparkling wines, the continuous homogenization of the liquid in the tank in the Charmat method and the permanence of the bottles static with the lees concentrated at the bottom in the Traditional method affect the yeast behaviour. During the ageing process, the constant homogenization of the sparkling wine (Charmat method) that maintained yeast cells in suspension determines a faster reduction in yeast vitality and viability compared with the ageing in the static Traditional method. Along with the onset of this accelerated viability decline due to tank agitation, there is an increase in the expression of autophagy markers, indicating that this process occurs early. Taken together, these results indicate a significant difference between the behaviour of “planktonic” and “benthic” cells during the long starvation period of sparkling wine ageing. However, despite these differences, the evolution of the wines in both methods is similar, showing that the practical results can be similar in both methods.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2022.104171.

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4.3 Capítulo 3

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ORIGINAL RESEARCH ARTICLE

Are the characteristics of sparkling wines obtained by the Traditional or Charmat methods quite different from each other?

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ABSTRACT

In this study, we performed an analytical and sensorial comparison between sparkling wines produced by the Traditional and Charmat methods using the same base wine, yeast strain, inoculum, and aged on the lees during the same periods. The absence of evident differences in the results of the analyses of physicochemical and volatile compounds was confirmed by the sensory analysis. In general, during the tests, more evaluators could identify differences in the first stages in which sensory analyses were performed. As the ageing time on the lees increase, fewer evaluators could differentiate between the sparkling wines. It was observed that more than half of the evaluators could not differentiate the samples in all stages. Based on our data, we conclude that the method used for the second fermentation is not the determinant of the eventual differences currently associated with sparkling wine produced by the Traditional and Charmat methods.

KEYWORDS: volatile compounds; sensory analysis; triangle test; qualitative similarities

INTRODUCTION

Sparkling wines are produced in different regions around the world, and their production involves different grape varieties, which are associated with edaphoclimatic characteristics, and different winemaking methods, which determine their specificities and, eventually, geographic names (appellation of origin). Sparkling wines can have different concentrations of residual sugar, acidity, ethanol, and diluted carbon dioxide (Di Gianvito *et al*., 2019). Sparkling wine with two fermentations is the result of the refermentation of the base wine, and there are two main methods to conduct it: the Champenoise, Traditional, or Classic method, or the Granvas, Bulk, or Charmat–Martinotti method (commonly called only Charmat). In the Traditional method, the second fermentation of the base wine takes place in sealed bottles (Butnariu, 2020; Buxaderas *et al*., 2022), while in the Charmat method, the second refermentation takes place in an isobaric tank (Butnariu, 2020). The term Champenoise should only be used officially for sparkling wines produced within the Champagne denomination of origin in France (Council regulation (EEC) N° 3309/85 of 18 November 1985 and EEC N° 2333/92 of 13 July 1992).

Each method has its technological peculiarities. In the Traditional method, the second fermentation is conducted in small-volume glass bottles (normally 750 ml), and the bottles remain static in a horizontal position, with the lees decanted at the bottom of the bottle. Usually, a fining agent (clarifier) is added to facilitate the removal of the lees during the "*remuage* and *dégorgement*" (Di Gianvito *et al*., 2019; Togores, 2018). Conversely, in the Charmat method, the second fermentation takes place in large-volume pressure tanks, usually made of stainless steel. The tanks have an internal shaker that keeps the liquid homogeneous. Moreover, these wines are filtered before bottling and do not need the addition of clarifiers (Jackson, 2020; Togores, 2018). Regarding ageing on lees (yeast cells and other precipitates), normally, the sparkling wines made by the Traditional method age for longer periods (more than one year) compared with those produced by the Charmat method (less than six months). However, there is possible to extend the time of contact with the lees in the Charmat method, currently named "long Charmat" (Jackson, 2020).

It seems that, technically, the differences attributed to the method used during the second fermentation (prise de mousse or foaming) of sparkling wines are overestimated, as during this fermentation: (1) just 20 to 25 g/L of sugar is consumed, giving 1 to 1.5 % (v/v) ethanol; (2) aromatic precursors present in the grape juice were metabolised during the first fermentation, and are less available for further biotransformation; (3) yeast population is relatively low ($\leq 10^8$ cells/mL), and yeast aromatic contribution during ageing is controversial (Sawyer *et al*., 2021). Therefore, there are other factors before the second fermentation, which are much more significant in the overall "difference" currently attributed to the "sparkling wine method" used.

Over time, wine communication and marketing have emphasised "the better quality of Traditional sparkling wines" to the point that consumers disregard wines produced by other methods, even without trying them (Vecchio *et al*., 2018; Verdonk *et al*., 2021). Moreover, as happen in different wine categories, some wines are more prone than others to ageing and benefit more or less from this process (Ribéreau-Gayon *et al*., 2021). For this reason, and the commercial appeal, normally the best base wines, more suitable for ageing, are currently destinated to the Traditional method, leaving the younger and lighter wines to be used in the Charmat method. Given this fact, quantitative and qualitative comparisons between sparkling wine-making methods using commercial sparkling wines should be avoided (Culbert *et al*., 2017). In this case, the variable "winemaking method" cannot be considered an independent variable as it is not directly associated with the quality of the final product. However, what would happen if we used the same base wine and inoculum in both methods and aged them for the same period? Taking this question in mind, we evaluate sparkling wines produced on an industrial scale using the same base wine, yeast strain, and inoculum and fermented by Traditional and Charmat methods. To compare these wines, we evaluate their physicochemical parameters, volatile composition, and sensorial attributes.

MATERIALS AND METHODS

1. Yeast Inoculation

The yeast *Saccharomyces cerevisiae* strain LALVIN® EC1118 (Lallemand, Canada) in active dry form was used in all the experiments. After hydration (manufacturer's protocol), the adaptation to the alcoholic medium by a gradual increment of ethanol- base wine (1st day 25 % wine/water; 2nd day 50 % wine/water; 3rd day 75 % wine/water, and from 4th day just wine), during yeast multiplication. Sugar concentration was kept near 15 g/L, and readily yeast assimilable nitrogen (YAN) was maintained near 0.75 g/L (35 % yeast extract and 65 % ammonium phosphate dibasic). This process was conducted in a specific tank with a temperature control system (12 °C), homogenisation (20 min of homogenisation followed by 80 min without homogenisation), and aeration (3 min compressed air injection followed by 180 min without injection). The percentage of ethanol at the time of inoculation was 13.3 % v/v, and the total yeast population was 3.5×10^7 cells/mL with a viability of 70 %. The base wine was inoculated with 1.4×10^6 viable cells/mL, representing 4 % (v/v) of the final fermentation volume.

2. Base wine and tirage

The second fermentation in both Charmat and Traditional methods was conducted at the Chandon of Brazil winery (Garibaldi, RS, Brazil) on an industrial scale. The base wine of sparkling wine used was a blend (*assemblage*) of Chardonnay (36 %), Riesling Italic (30 %), and Pinot noir (34 %) vinified in white. The base wine had: 1 g/L of reducing sugars; 11.2 % alcohol (v/v); 6 g/L of total acidity (expressed in tartaric acid); a pH of 3.27; 0.28 g/L of volatile acidity;

14 mg/L of free sulfur dioxide (SO_2), and 80 mg/L of total SO_2. Before the second fermentation, the base wine was submitted to a tartaric stabilisation for 20 days at –2 ± 1 °C and then filtered (tangential filter Bucher Vaslin—Flavy FX 06, France).

The base wine, 22 g/L sucrose, and yeast inoculum (4 % v/v) were transferred to a vertical pressure tank (50,000 L) equipped with a rotating stirrer. Part of the wine was bottled for the Traditional method in specific sparkling wine bottles with a volume of 750 ml and was added 3 g/hL of clarifiers (bentonite + alginate; CLEANSPARK—Laffort, France) to help remove the yeasts after fermentation. The closure of the bottles was made with a plastic *bidule* and a metal crown (stainless steel) TOP+ (PE.DI, Italy). The rest of the wine remained in the pressure tank (Charmat method) without the addition of clarifiers.

3. Second fermentation, ageing of sparkling wines and sample collections

The second fermentation, in both methods, was conducted at a temperature of 12 ± 1 °C. It was considered the end of fermentation when the amount of reducing sugars was below 3 g/L, finished after 11 weeks (77 days). During the maturation period on the lees, the temperature of the tank (Charmat method) was reduced to 6 °C (a temperature used by the winery), and the bottles (Traditional method) were kept at 10 °C, a temperature commonly used in the Traditional method (simulating an ageing cellar). In the Charmat method, the wine was continuously homogenised by an internal propeller at 60 rpm throughout the fermentation period and subsequent ageing. Conversely, in the Traditional method, the bottles remained immobile during the whole period.

During the second fermentation, samples were collected weekly (three bottles and approximately 1.5 L from the pressure tank). During this period, physical and chemical analyses for fermentation monitoring were performed. After the end of fermentation at the times of 4, 9, 12, 16, and 22 months of ageing, the sparkling wines were prepared for sensorial and chemical analyses. In the Traditional method, *remuage* and subsequent *dégorgement* were performed. In the Charmat method, an isobaric filtration (0.45 µm membrane filter) was performed, followed by the bottling. At each time, approximately 20 bottles of each method were prepared. In both methods, 50 mg/L of SO_2 was added at the time of corking. Sugar (expedition liqueur) 10 g/L (Brut) was added only at 22 months in a portion of the bottles in both methods. In this case, in the Charmat method, the sparkling wine was filtered with a tangential filter (Bucher Vaslin—Flavy FX 06), after which the expedition liqueur was added to the pressure tank, and then the sparkling wine was filtered again with a plate filter (cellulose plates) and then with a membrane filter (0.45 µm) before bottling. In the Traditional method, post *dégorgement*, the process of adding the expedition liqueur was conducted manually. After corking, the sparkling wines remained in an upright position in an ageing room at a temperature between 16–20 ºC. In all stages, the sensory analyses were conducted between 3 and 4 months after corking.

4. Oenological analysis

The wine density was performed by direct reading with a specific gravity hydrometer (0.900/1.000 scale) and was expressed in g/L. Reducing sugars (hydrolysed) were quantified by the modified Lane–Eynon procedure (Zoecklein *et al*., 1990), which is based on the property of sugars to reduce alkaline copper sulfate under specified heating conditions. The values found were expressed in g/L of reducing sugars. The pressure level inside the bottles was measured using an aphrometer and in the tank using a manometer and was expressed in atmospheric pressure (atm) at 20 ºC.

Ethanol was determined by distillation (Super Dee Digital Distilling Unit - Gibertini, Italy) and measurement of the distillate density at 20 °C using an alcoholmeter. The alcohol was expressed by volume-volume percentage (% v/v). The determination of pH was performed with a Thermo Electron Orion Model 310 pH meter (MA, EUA), and total acidity (TA) was measured by titration with 0.1 N sodium hydroxide solution using bromothymol blue as an indicator (OIV, 2015). Results were expressed in g/L tartaric acid.

Volatile acids evaluation was done by steam distillation in Super DEE (Gibertini, Italy) and quantified by titration (OIV, 2009). Volatile acidity was expressed as g/L of acetic acid. The concentration of free and total SO_2 was measured using colorimetric titration by the Ripper method (Adams, 1988). The result was expressed in mg/L.

The yellow colour (Abs 420 nm), tint (Abs 420 nm/ Abs 520 nm), and colour intensity (Abs 420 nm + Abs 520 nm + Abs 620 nm) of wines were determined by absorbance using a spectrophotometer (Pró-tools UV–1600—Shanghai Mapada Instruments, China) and expressed as optical density units (Ribéreau-Gayon *et al*., 2021). Turbidity was evaluated by a turbidimeter (2100P—Hach, USA) and expressed in NTU (Nephelometric Turbidity Units).

5. Analysis of volatile compounds

Volatile compounds were analysed at 0 (base wine), 4, 12, and 22 months. Extraction of volatile compounds was performed in triplicate (three bottles of each method) using solid phase microextraction (SPME) with polyacrylate fibre Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS—50/30 µm) (Supelco, Sigma-Aldrich, USA), according to the adapted methodology (Xiao *et al*., 2015). Where in 20 ml headspace vial with silicone septum was added, 8 ml of foaming sample, 2 g of sodium chloride, and 80 µL of 3-octanol (10 mg/L) as standard compound. In a thermostatic bath, the fibre was exposed to the space above the liquid (headspace), and the sample was magnetically stirred at 50 ºC for 50 minutes.

After extraction, the fibre was applied to the injector of the GC/MS apparatus. A gas chromatograph (GC) 6890 coupled to a mass selective detector (MS) 5973 (Agilent Technologies, USA) was used, with an HP-INNOWAX column (30 m × 0.25 mm × 0.25 µm). Oven conditions were 40 °C for 2 min, increasing at a rate of 3 °C/min to 230 °C for 2 min.

The injector temperature was set to 230 °C, and the fibre was injected in desorption permanence for 5 min in split-less mode with helium at a constant flow rate of 1.2 mL/min. MS parameters included electron impact ionisation with electron energy of 70 eV and mass range of m/z 30-550, using ion-selective monitoring (SIM) mode. The area of each peak was determined by ChemStation software (Agilent Technologies). Identification of the compounds was obtained by comparing the retention index (RI) with those reported in the literature and the fragment mass patterns with those in the Wiley (Hewlett-Packard, Palo Alto, CA) and NIST Database. Quantification of the compounds was performed by comparing the area of the compounds with the area of the internal standard (3-octanol).

6. Sensory analysis

During the sensory analyses and after the end of the research, all ethical-legal precepts were maintained, according to Resolution 466/2012 of the National Health Council (Brazil). The project was submitted and approved by a research ethics committee of the Federal Institute of Education, Science and Technology of Rio Grande do Sul (reference number: 3.622.321).

6.1. Triangle test

The forced-choice discriminatory method, the ISO 4120 triangle test, was used to compare the differences between the two sparkling winemaking methods (ISO, 2004). In all stages of sensory analysis, each evaluator performed the test only once. The evaluators had sufficient knowledge and sensory acuity to discriminate the samples at the desired level (oenologists, oenology teachers, viticulture and oenology students, sommeliers, and other professionals in the beverage and food sector). Among the judges, 58 % were men and 42 % women, with an average age of 30 years (youngest 18 years and oldest 70 years). The tests were conducted in 5 stages, with a total of 369 tests applied. The sensory analyses took place in specific rooms following ISO 8589 (ISO, 2007). The sparkling wines were served in ISO glasses (40 ml in each glass) with three samples simultaneously at a temperature of 8 °C. Each participant received full instructions on the operation of the test before the start of the evaluation as described in ISO 4120. At all stages, the samples were served in presentation order, followed by a sorting protocol with a balanced, randomised design.

The results of the triangle test assume a binomial distribution (0, the taster did not correctly identify the different sample; 1, the taster correctly identified the different sample). The results of each stage were obtained based on the number of right and wrong answers to the total number of evaluators and are presented in Figure 3 with relevant information for each stage in which the tests were performed. In all stages, a level of statistical rigour was defined with a low risk of concluding that there are differences when there is not (type I or α error), being $\alpha = 0.05$ with a 95 % probability of detecting differences between the samples. To verify whether the number of hits for each stage obtained significant differences, the tables and formulas described in the ISO 4120 method were used. Furthermore, the confidence intervals were calculated (bilateral with a critical value of 95 %) for the proportion of the population that can discriminate the samples in each evaluation step.

6.2. Quantitative descriptive analysis (QDA)

A quantitative descriptive analysis adapted from the standards described in ISO 8586 was performed (ISO, 2012) to quantify the typical characteristics of sparkling wines at the last collection point (22 months) with samples without expedition liqueur (Nature) and with expedition liqueur (Brut—10 g/L of sugar) in both methods. For this analysis, an expert panel (oenologists working in sparkling wine production) of 12 judges (7 men and 5 women) was assembled. The intensity of each attribute was assessed

TABLE 1. Order of the samples evaluated in the Quantitative Descriptive Analysis (QDA).

Types (Sweetness)	Order	Method	Ageing temperature
*Reference sample	1	Charmat	-
Nature	2	Traditional	ageing at 10 °C
	3	Traditional	ageing at 6 °C
	4	Charmat	ageing at 6 °C
	5	Traditional	ageing at 6 °C
	6	Charmat	ageing at 6 °C
	7	Traditional	ageing at 10 °C
Brut	8	Charmat	ageing at 6 °C
	9	Traditional	ageing at 10 °C
	10	Traditional	ageing at 10 °C
	11	Charmat	ageing at 6 °C

*Reference sample = commercial sparkling wine, long Charmat Extra Brut; Nature = without added expedition liqueur; Brut = addition of 10 g/L sugar.

using a 12 cm unstructured line scale labelled as "low" at the left end and "high" at the far right of the line. The global descriptors defined were visual, colour intensity, in the aromas, genuineness, positive intensity, tropical fruits, citrus fruits, dry fruits, flowers, spice, bread dough, herbaceous/vegetable, defects, and the taste sensations, Genuineness, positive intensity, body, sweetness, acidity, persistence, harmonious, bitterness, defects, and Quality (general). Among these attributes, those related to aromas of spice, herbaceous/vegetable, aromas defects, and taste defects were not presented in the results because they were not detected by the participants (values lower than 0.5 on a scale up to 10).

To evaluate the sparkling wines, ISO glasses were used (40 ml per glass). The samples were tasted "blind" at individual tables under artificial lighting with white LED lamps. The wines were served individually and randomly (defined by lottery), separated into Nature and Brut with one repetition. Before starting the analysis, a joint evaluation was made with the participants, where a commercial sparkling wine (reference sample, commercial sparkling wine, long Charmat Extra Brut) was analysed, and the characteristics of the product were discussed among the judges to present the evaluation form, answer questions and balance opinions. The service temperature of the sparkling wines was controlled at 8 °C. In total, 11 samples were analysed in the order described in Table 1.

The results obtained were submitted to a parametric statistical analysis as described below (item 3.7). The results of the sample with the Traditional method aged at 6 °C (the same ageing temperature as the Charmat method) were not presented as they did not differ from the other sparkling wines.

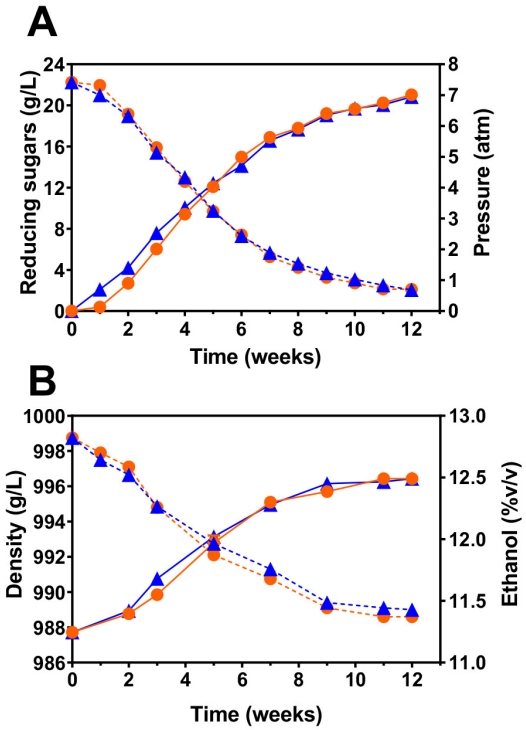
7. Statistical analysis

Statistical analyses comparing treatments at each time point during fermentation and ageing were performed with two-tailed unpaired t-tests with a P value of less than 0.05 was considered significant. In the results referring to the triangular test, the statistical analyses were performed following the models and tables available in the ISO 4120 standard (ISO, 2004). In the quantitative descriptive analysis and analysis of volatile compounds, the one-way ANOVA statistical test was performed, followed by the Tukey test, with a p-value less than 0.05 considered significant.

RESULTS

1. Second fermentation of sparkling wine

The kinetics of the second fermentation of the sparkling wines using the Charmat or Traditional method were similar (Figure 1). This shows that the control and homogeneity of the processes were efficient, which helps to make our sensory and analytical comparison valid. Moreover, the



C

End of fermentation

Analysis	Traditional	Charmat
pH	3.3±0	3.3±0
Total acidity (g/L)	3.9±0	3.9±0
Volatile acidity (g/L)	0.3±0	0.3±0
SO_2 free (mg/L)	5.3±0.2	5.3±0.2
SO_2 total (mg/L)	70±1	74±1
Abs 420 nm	0.102±0.001	0.100± 0.001
Abs 520 nm	0.026±0.001	0.025±0.001
Abs 620 nm	0.007±0,001	0.006±0.001
CI*	0.134±0.003	0.131±0.003
Tint**	3.9±0.02	4.08±0.08

FIGURE 1. Monitoring of fermentations in the two methods conducted at 12 °C.

A = Reducing sugars (dotted line) x increasing pressure - atmospheric pressure (atm) measured at 20 °C; B = Decrease in density measured at 20 °C (dotted line) x increased ethanol concentration; C = table with analyses at the end of fermentation. * CI = Colour intensity (Abs 420+520+620); Tint** = (Abs 420/520); Error bars in line graphics represent standard deviation (SD). Table values are presented as mean ± SD. Both SD were obtained from triplicate samples (3 bottles and 3 tank samples) within the same experiment. Single comparisons were performed between the methods with unpaired two-tailed t-tests between treatments at each stage at which sensory analysis was performed. No significant differences were found.

addition of clarifiers (bentonite + alginate) in the Traditional method did not influence the fermentation kinetics and the physicochemical characteristics of the wines (Figure 1). Overall, there was no significant difference between the methods in the physicochemical parameters at the end of the second fermentation (Figure 1C).

2. The evolution of sparkling wines during ageing

2.1. Physicochemical parameters

Table 2 shows the physicochemical analyses commonly performed for wine quality control. These analyses were performed after 4, 9, 12, 16, and 22 months, the same periods in which the sensory analyses were performed. As can be seen in Table 2, there were slight differences between the wines obtained by the Charmat and Traditional methods during ageing. Significant differences between the methods were detected only in the sugar concentration that was higher in Charmat sparkling wines, the concentration of SO_2 and tint at some point during ageing, and the turbidity that was higher in Charmat wines at 9 and 12 months. These differences in turbidity are related to the difference between the filtration performed before bottling in the Charmat method and the *dégorgement* in the Traditional method.

Parameters like ethanol concentration (12.4 ± 0.2 %), total acidity (3.9 ± 0.1 g/L), pH (3.3) and volatile acidity (0.3 ± 0 g/L) did not show significant differences between processes or along maturation. In the same way, the yellow colour (Abs 420 nm) and colour intensity did not vary significantly. Regarding the internal pressure of the sparkling wines, there were no significant differences between the methods. Care was taken at all stages of corking to ensure that the pressures were kept close together and did not impair the sensory analysis. All the values are within the quality standards used to classify this type of product (Togores, 2018).

2.2. Volatile compounds

The volatile compounds found, which are responsible for the aromas of the sparkling wines, had almost no significant variations between the methods (Figure 2). The only compound that varied between the two methods was diethyl succinate, which had an early increase in the Traditional method, while the other compounds maintained their concentration over time.

The greatest differences occurred between the base wine and sparkling wines regardless of the method and the maturation on their lees (Figure 2). After the second fermentation, there

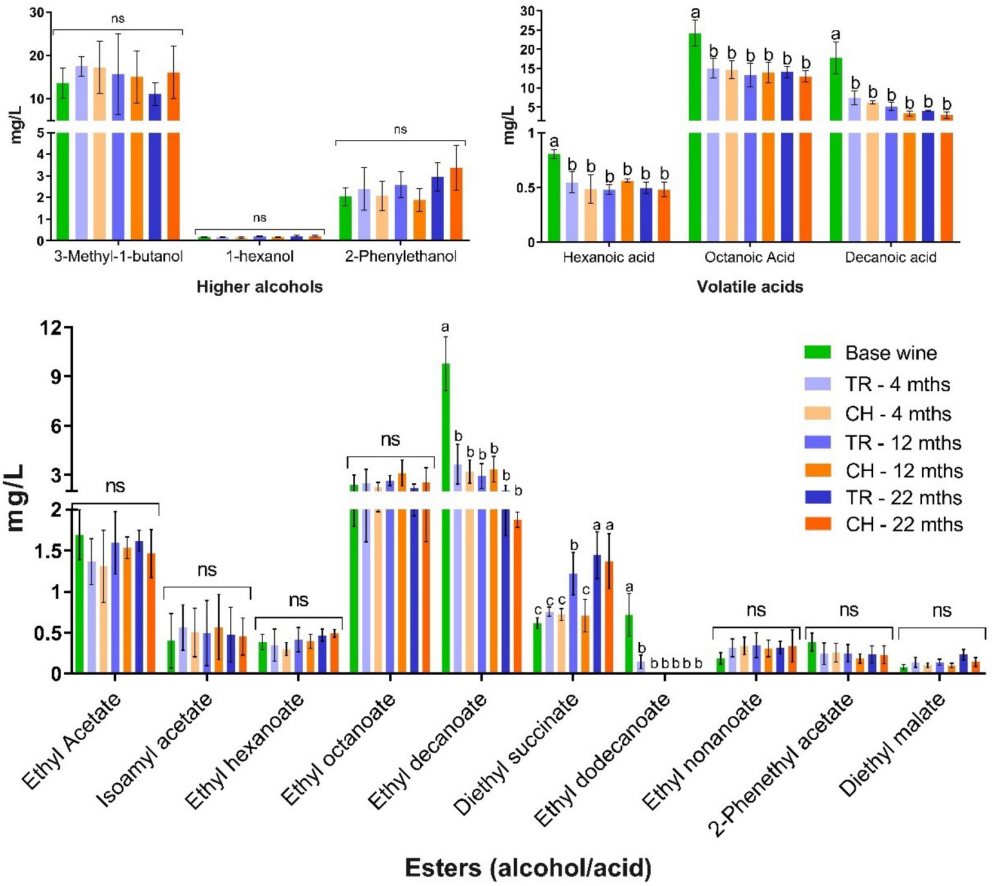


FIGURE 2. Analysis of volatile compounds. Gas chromatography with solid phase microextraction (SPME) with polyacrylate fibre. Different letters at each time indicate statistical differences in One-way ANOVA followed by Tukey's test ($p < 0.05$). ns = not significant.

TABLE 2. Oenological analysis of sparkling wines (means ± standard deviation).

Time with lees	4 months		9 months		12 months		16 months		22 months		22 months (Brut)	
Method	Charmat	Traditional	Charmat	Traditional	Charmat	Traditional	Charmat	Traditional	Charmat	Traditional	Charmat	Traditional
Ethanol (% v/v.)	12.3 ± 0.1	12.4 ± 0.1	12.5 ± 0.1	12.5 ± 0.1	12.4 ± 0.1	12.4 ± 0.1	12.4 ± 0	12.5 ± 0.1	12.5 ± 0.1	12.4 ± 0.1	12.2 ± 0.1	12.1 ± 0.1
Reducing sugars (g/L)	2.1 ± 0*	1.5 ± 0*	1.9 ± 0*	1.3 ± 0.1*	1.9 ± 0*	1.2 ± 0.1*	2.1 ± 0.1*	1 ± 0*	1.8 ± 0.2*	1 ± 0*	10 ± 0.2	10 ± 0.1
Pressure (atm at 20 °C)	6.1 ± 0.1	6.5 ± 0.1	5.8 ± 0.2	5.8 ± 0.1	5.8 ± 0.1	6.2 ± 0.2	6.9 ± 0.1	6.8 ± 0.2	6.3 ± 0.1	6.5 ± 0.1	5.8 ± 0.1	6.3 ± 0.2
pH	3.3 ± 0	3.3 ± 0	3.3 ± 0	3.3 ± 0	3.3 ± 0	3.3 ± 0	3.3 ± 0	3.3 ± 0	3.3 ± 0	3.3 ± 0	3.3 ± 0	3.3 ± 0
Total acidity (g/L)	3.9 ± 0	3.9 ± 0	3.9 ± 0	3.9 ± 0	3.9 ± 0	3.9 ± 0	3.9 ± 0	3.9 ± 0	3.8 ± 0	3.9 ± 0	3.8 ± 0	3.9 ± 0.1
Volatile acidity (g/L)	0.3 ± 0	0.3 ± 0	0.3 ± 0	0.3 ± 0	0.3 ± 0	0.3 ± 0	0.3 ± 0	0.3 ± 0	0.3 ± 0	0.3 ± 0	0.3 ± 0	0.3 ± 0
SO_2 free (mg/L)	13.2 ± 2.5	14.8 ± 1.1	10.3 ± 1.2*	15.7 ± 0.6*	9.8 ± 0	12.6 ± 1	8.3 ± 0.3*	13.9 ± 1*	13.4 ± 1.5	14.1 ± 0	10.3 ± 1.5	12.3 ± 0.6
SO_2 total (mg/L)	108.8 ± 3.8	108.8 ± 2.5	97.7 ± 1.5*	107.3 ± 2.5*	99.1 ± 1.4	102.4 ± 4.9	99.1 ± 2.2	105.9 ± 5.5	106.5 ± 1	105.2 ± 2	102 ± 1	103.6 ± 2.3
Abs 420 nm	0.102 ± 0	0.109 ± 0	0.103 ± 0	0.104 ± 0	0.106 ± 0	0.103 ± 0	0.107 ± 0	0.111 ± 0	0.098 ± 0	0.107 ± 0	0.105 ± 0	0.120 ± 0
Abs 520 nm	0.028 ± 0	0.034 ± 0	0.030 ± 0	0.025 ± 0	0.034 ± 0	0.027 ± 0	0.031 ± 0	0.028 ± 0	0.024 ± 0	0.021 ± 0	0.028 ± 0	0.033 ± 0
Abs 620 nm	0.009 ± 0	0.015 ± 0	0.011 ± 0	0.006 ± 0	0.016 ± 0	0.006 ± 0	0.013 ± 0*	0.007 ± 0*	0.006 ± 0	0.010 ± 0	0.008 ± 0	0.010 ± 0
Colour Intensity (Abs 420+420+620)	0.139 ± 0	0.157 ± 0	0.145 ± 0	0.135 ± 0	0.156 ± 0	0.137 ± 0	0.151 ± 0	0.145 ± 0	0.127 ± 0	0.138 ± 0	0.142 ± 0	0.164 ± 0
Tint (Abs 420/520)	3.6 ± 0.3	3.2 ± 0	3.4 ± 0.2*	4.2 ± 0.1*	3.2 ± 0.3	3.8 ± 0.2	3.4 ± 0.2	4 ± 0.1	4.1 ± 0.4	5.1 ± 0.1	3.8 ± 0.2	3.6 ± 0.1
Nephelometric Turbidity Unit	1 ± 0.1	0.9 ± 0.1	1.9 ± 0.1*	0.8 ± 0.1*	2.2 ± 0.3*	0.8 ± 0*	0.8 ± 0.1	1.1 ± 0.1	1.2 ± 0.2	1 ± 0.1	0.6 ± 0.1	1.2 ± 0.2

Single comparisons were performed between the methods with unpaired two-tailed t-tests between treatments at each stage (* significant differences $p < 0.05$).

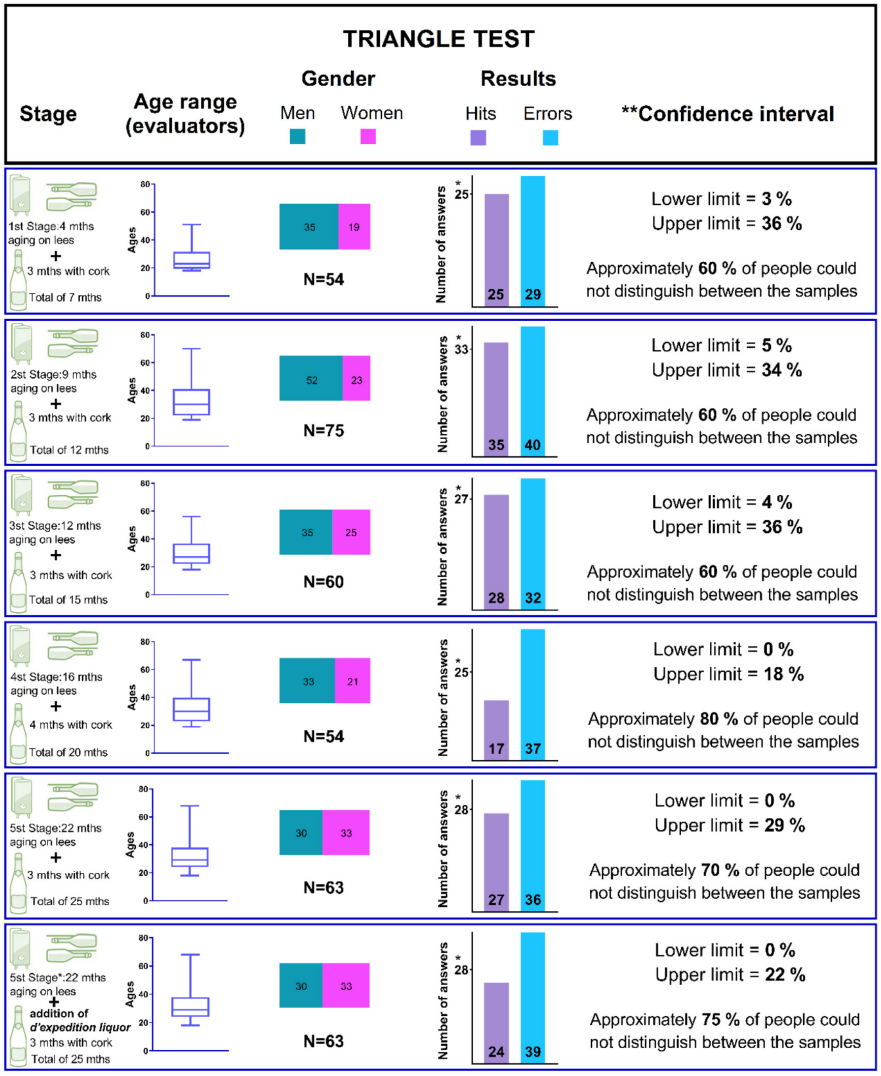


FIGURE 3. Triangular test conducted at different ageing times of sparkling wines. All statistical results were obtained based on ISO 4120 (ISO, 2004).

N = the number of evaluators in each period. Stage = time of contact with lees and time whit cork. *Critical value of correct answer = the minimum number of correct answers to conclude that there are differences at the tested level, 95 % confidence level whit α risk of 5 % to 1 % (indicates moderate evidence that the difference was apparent). **Confidence interval = (95 % confidence level) the proportion of the population that can distinguish the samples is between the lower and upper confidence limit.

was a significant reduction in the concentration of volatile acids: hexanoic acid, octanoic acid, and decanoic acid, which a responsible for leafy, wood, vanish, butter, almond, and caramel aromas, as well as the concentration of decanoate and dodecanoate ethyl esters, that are described to contribute with fruity, fatty, sweet, floral, cream, and other pleasant and fresh aromas (Hu *et al.*, 2018). Interestingly, we did not detect a significant reduction in the concentration of acetate esters or an increase in higher alcohols during ageing in either method. The absence of significant differences between the sparkling wines obtained by the Charmat and Traditional methods showed that the method did not influence these parameters.

3. Sensory evaluation

Figure 3 shows the results of all periods in which the sensory analyses were performed regarding the triangle test. As can be observed, the data shows the ageing time of the sparkling wines, the average age, gender, and the number of correct and incorrect answers of the participants in each period. Next to each tasting time is a summary of the statistical analysis and the result of the confidence interval calculation in percentage.

In general, the data showed that there is a higher percentage of correct answers and a higher confidence interval (higher number of judges capable of distinguishing the samples) in the first months in which the test was applied (4, 9, and 12 months of contact with lees). However, in the tests applied after one year (16 and 22 months of contact with the lees), the percentage of judges who were able to distinguish between the samples decreased together with the confidence interval. In all stages, more than half of the judges were not able to differentiate the sparkling wines and distinguish the elaboration methods. The addition of expedition liqueur at 22 months seemed to make it even more difficult for the judges to differentiate the samples.

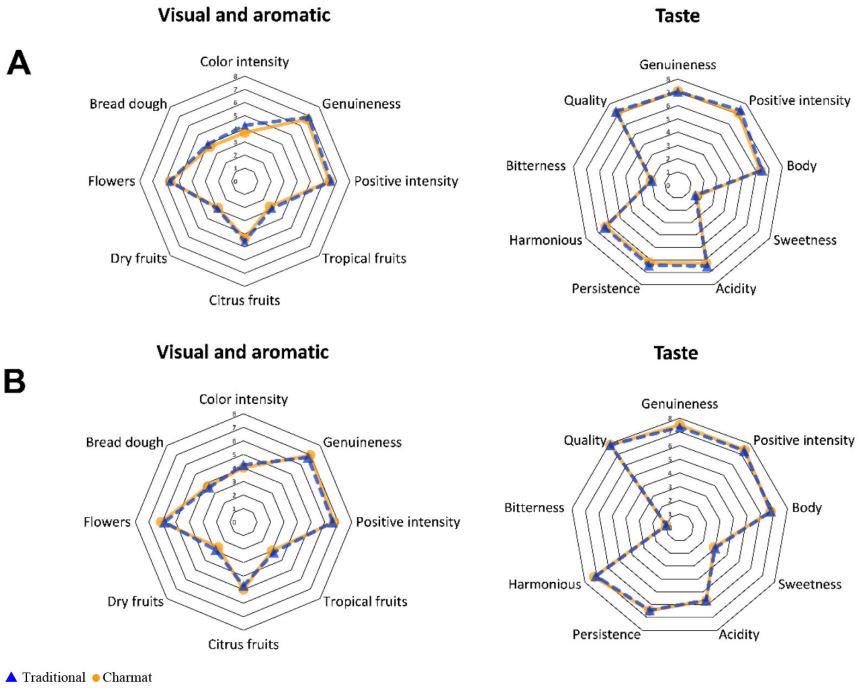


FIGURE 4. Quantitative Descriptive Analysis (QDA).

A = Sparkling nature (without expedition liqueur); B = Sparkling Brut (dosage of 10 g/L of sugar in the expedition liqueur). The results were obtained by a trained panel of 12 tasters. The graphics were separated into visual, aromatic, and taste. There were no significant differences between the method (One-way ANOVA followed by Tukey's test $P < 0.05$).

In the last stage of sparkling wine evaluation (22 months of contact with the lees), a panel of trained tasters (oenologists specialising in sparkling wine elaboration) performed a quantitative analysis of the attributes commonly described in sparkling wines (Figure 4). In all the attributes quantified, the two methods did not present differences that were possible to discriminate between the elaboration methods, the values on most attributes being close. The only differences found were between sparkling wines with and without expedition liqueur, regardless of the method. The sparkling wines with the addition of liquor were considered sweeter, less acidic, more harmonious, and of better quality.

DISCUSSION

Although the methods of making Traditional or Charmat sparkling wines have technological characteristics, the physicochemical, volatile, and sensory differences of sparkling wines are controversial. Our study shows that the sparkling wines produced from the same base wine, inoculum, and ageing time on lees, do not differ much in their chemical (Figures 1 and 2, and Table 2) and sensory (Figures 3 and 4) characteristics to the point of being able to differentiate or identify the method that was used.

Even though are already some studies in the literature on the same subject with contrary conclusions, they all differ from our study in that either the comparisons between the methods were made with commercial sparkling wines (Culbert *et al*., 2017; Ubeda *et al*., 2016), or the sparkling wines had different fermentation temperatures or different contact times with the lees depending on the method (Caliari *et al*., 2015; Vecchio *et al*., 2018), which makes it difficult to compare the methods properly. In our study, the comparison between the Traditional and Charmat methods sought to respect as much as possible the techniques normally applied in each of the methods on an industrial scale, the main variable being the method used to conduct the second fermentation. The base wine, inoculum, and fermentation temperature form the same, and the two methods have similar fermentation kinetics (Figure 1). Despite the differences in ageing temperature between the methods in our study (Charmat method of 6 °C and Traditional methods of 10 °C), during the follow-up time of our study (approximately 2 years), we saw that this difference did not cause noticeable changes in the overall characteristics of the products. It is interesting to comment that in one study evaluating the same base wine aged with and without contact with the lees and the sparkling wine from this base wine aged on its fewer for 24 months (Sawyer *et al*., 2021), the researchers found no significant differences in the aromatic profile of the wines, concluding that the quality of the base wine used and the oxy-reductive phenomena occurring during ageing are more important in this process than ageing on lees. According to our results, this is also true for the method of making Charmat and Traditional sparkling wines for a period of fewer than 24 months. For, in our comparison using the same base wine for the two methods, we identified few differences in the volatile compounds, where only the compound diethyl succinate varied statistically over time at 12 months (Figure 2). Whereas in a study conducted in Chile (Ubeda *et al*., 2016) comparing the aromatic profile of commercial sparkling wines

made by the Charmat and Traditional methods (16 Chilean sparkling wines, 8 of each method), the main difference between the sparkling wines was the higher presence of ethyl esters in Traditional method sparkling wines and higher amounts of acetic esters and ketones in Charmat sparkling wines. These two classes of esters (ethyl esters and acetate esters) are respectively related to characteristics of aged wines and young wines (Waterhouse *et al*., 2016). This case shows an example of the types of products that are normally made with each method (young Charmat sparkling wines and Traditional aged sparkling wines), but by no means do these characteristics come exclusively from the type of method used to conduct the second fermentation of the sparkling wines.

An important fact that occurs when we talk about sparkling wine-making methods is the expectation effect that occurs whit the people. An interesting study was conducted to understand the effect on quality expectations and how information about the method by which the sparkling wine was made influences the consumer (Vecchio *et al*., 2018). Although the sensory comparisons between the methods were made with sparkling wines fermented at different temperatures and with different times of contact with the lees (Traditional 15 months in contact with lees Charmat 4 months in contact with lees), which may impair the comparison between the methods, the researchers carried out a hedonic evaluation of the products without tasting, only through the label with detailed information about each production method. Sparkling wines produced by the Traditional method were preferred in this hedonic choice. In this case, the information about the method created expectations of taste and quality without the assessors having tried the products. This may be because the Traditional method is related to "Champagne" sparkling wines, which have a formidable reputation acquired over time and a strong collective territorial brand (Charters and Spielmann, 2014), which is probably why to this day, there is this myth that one method is better than the other.

In our sensory analyses concerning the triangle test over time, we saw that a greater number of judges were able to identify the samples in the first months of evaluation (Figure 3). It may be that in this period, the autophagic and autolytic phenomena of yeasts that differ between the methods (Cisilotto *et al*., 2023), may modify some organoleptic sensations. However, in any case, if there are slight differences, they are very subtle because, in addition to the chemical analyses being similar (Figure 1 and 2), in all the tastings, more than half of the judges were not able to identify the different sparkling wine (Figure 3). Added to this, we saw that in the quantitative descriptive sensory analysis conducted with the professionals (Figure 4), the characteristics of the sparkling wines that were quantified did not have statistical differences, which further strengthens our argument that the method does not define the main characteristics and quality.

All these results show that although there is marketing that praises the quality of sparkling wines made with the Traditional method, sparkling wines made with the Charmat method can have the same level of quality and ageing capacity. However, in most cases, sparkling wines made using the Charmat method go to market much younger than those made using the Traditional method, assuming this identity and relation with young and fruity products. In our understanding, the quality of the base wine used plays a key role in this process, and with both methods, it is possible to make both sparkling wines with characteristics of young and fruity wines, with less time of contact with the lees, or more aged with characteristics related to the evolution of wines. This is a fact known by many winemakers, but this knowledge is still not clear to many professionals and especially to the final consumer.

Our study is the first one that used the same base wine and inoculum and treated the ageing time with the lees equally in both methods, performing regular tastings with care to be as faithful as possible to the industrial reality. Under these conditions presented, in general, the methods are similar, having a similar behaviour over time, and as we have seen, most people cannot differentiate the method employed in the second fermentation of sparkling wines. This shows that regardless of the method used to conduct the second fermentation of the sparkling wines, in both methods, it is possible to obtain similar sparkling wines and that the methods do not define the main characteristics and the final quality of the sparkling wines.

CONCLUSIONS

From the same base wine and inoculum, with the wines ageing the same amount of time on their lees, the sparkling wines made by the Charmat and Traditional methods are similar in every aspect. The differences found in sparkling wines made by Charmat and Traditional methods are subtle and do not cause major changes capable of modulating the overall characteristics of the products. The longer the sparkling wines age, the more difficult it becomes to only differentiate the products sensorially by the method used in the second fermentation. The method used to perform the second fermentation does not define the quality of the sparkling wines, and it is possible to assume that there are other factors that precede the second fermentation that can have more impact on the product.

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5 Discussão Geral

Podemos considerar que soma dos nossos estudos poderá contribuir com os avanços da ciência, principalmente dentro das áreas de Biotecnologia, Microbiologia e Enologia. Partindo de uma pergunta controvérsia dentro da enologia (existe diferenças significativas entre os métodos de elaboração de espumantes Charmat e Tradicional, através do acompanhamento analítico e coleta de dados ao longo do tempo, além de responder à pergunta inicial, foi possível formular uma série de hipóteses e *insights*, além de novos objetivos a medida em que avançamos nos resultados.

No nosso primeiro estudo (Capítulo 1), formulamos a hipótese (ocorre um sinergismo entre o etanol e o dióxido de enxofre e isso aumenta o estresse das leveduras) durante a coleta de dados de viabilidade nos dois métodos de elaboração de espumantes. Os dois métodos não tiveram diferenças entre si, porém nos chamou a atenção que após a inoculação das leveduras ocorreu uma queda considerável da viabilidade. Pensando por que isso aconteceu e somando as dificuldades já descritas que existem nessa etapa (Benucci et al., 2016; Borrull et al., 2016; Martí-Raga et al., 2016), resolvemos testar a nossa hipótese. Em um primeiro momento parece óbvio que a presença desses dois compostos possam aumentar o estresse das leveduras, e de fato alguns autores já haviam demostrado esse efeito em vinhos estocados com leveduras de deterioração (Chandra et al., 2015, 2014; Edwards and Oswald, 2018), porém ninguém havia ainda testado em leveduras vínicas utilizadas especificamente para a segunda fermentação de espumantes, pois como se sabe, essas cepas possuem mecanismo específicos para lidar com a presença de dióxido de enxofre (Divol et al., 2012).

Além de mostrarmos o efeito sinérgico do Etanol e SO_2 em ambiente fermentativo, propusemos o mecanismo pelo qual isso ocorre, onde a presença de etanol modifica a permeabilidade da membrana plasmática (Aguilera et al., 2006; Madeira et al., 2010; Tesnière, 2019) e consequentemente há uma maior quantidade de SO_2 que consegue entrar no meio intracelular. Essa teoria pode ser sustentada com os nossos resultados onde vimos uma diminuição do pH intracelular, aumento de espécies reativas de oxigênio e aumento da expressão de genes relacionados com mecanismos de efluxo da molécula de SO_2 de dentro das células.

Os resultados e as conclusões em que chegamos com esse estudo podem servir de base para outros estudos, como por exemplo, pode ser testado o efeito sinérgico em diferentes cepas de leveduras, possibilitando trabalhos com melhoramento genético, pois provavelmente deve haver grande variação entre as espécies (Nadai et al., 2016). Outro estudo poderia testar

diferentes formas de adaptação do inóculo, pois, provavelmente deve haver formas em que a levedura se adapte melhor a presença dessas duas moléculas. Ao que parece, a constituição lipídica da membrana plasmática pode ser um fator chave nesse processo (Tesnière, 2019).

O que ficou evidente para nós foi que as doses de SO_2 no vinho base devem ser as mais baixas possíveis, porém nem sempre isso é praticável. Na verdade, o que vemos é uma tendência de aumento das doses, pois as mudanças climáticas estão tornando as uvas mais maduras e menos acidas (Venios et al., 2020). Entretanto, manter uma acidez elevada e um pH baixo é imprescindível para um espumante de qualidade (Jones et al., 2014). Isso faz com que os produtores de espumantes queiram evitar a fermentação malolática que é considerada uma desacidificação microbiológica, pois transforma o ácido málico em ácido lático, aumentando o pH e reduzindo a acidez total (Davis et al., 1985). Apesar de existir alternativas para evitar isso, o SO_2 tem ainda um papel muito importante nesse processo (Lisanti et al., 2019). De qualquer maneira, quanto mais baixas as doses de SO_2 livre e de etanol no momento da segunda fermentação menos estresse será causado nas leveduras e consequentemente, menor será o estresse do técnico responsável pelo processo.

Os resultados e os "caminhos percorridos" no estudo do Capítulo 2 da Tese inicialmente não eram esperados. Isso porque na coleta de dados realizada durante a segunda fermentação nos dois métodos, todas análises eram muito parecidas, tanto na cinética fermentativa, quanto no comportamento das leveduras e nas análises físico-químicas. Ao final da fermentação quando as quantidades de açúcares redutores eram mínimas, a morte acelerada das leveduras no método Charmat foi uma surpresa. No entanto, as tendências nas análises que mostravam uma queda mais acentuada na viabilidade foram se repetindo ao longo do tempo, o que nos levou a criar uma hipótese pensando no porquê isso estava acontecendo.

Eliminando todas as possíveis diferenças entre os métodos que poderiam ter alguma interferência, chegamos no fato de que no método Tradicional durante toda a fermentação e envelhecimento, as garrafas ficam imóveis na posição horizontal e as leveduras ficam depositadas acumuladas no fundo da garrafa, porém, no método Charmat o tanque de pressão conta com um agitador interno que possibilita que o líquido permaneça sempre homogêneo dentro do tanque. Essa diferença nos fez pensar na hipótese de que ocorreria uma retroalimentação no método Tradicional devido à proximidade das leveduras umas das outras, formando uma espécie de colônia, numa tentativa de sobrevivência a longo prazo. Ao contrário no método Charmat, com a homogeneização contínua do líquido isso não é possível. Para validar esse fenômeno repetimos essa parte do experimento com outro espumante com condições similares e o resultado foi similar, ocorrendo uma diferença na queda da viabilidade

separadas por meses entre as duas situações. Ao realizarmos as análises de expressão gênica mensurando as quantidades de RNAm, verificamos que com a homogeneização continua no método Charmat ocorre uma expressão gênica antecipada principalmente de genes relacionados com autofagia. Relacionamos esse fato como sendo uma provável tentativa de sobrevivência pela falta de possibilidades de obtenção nutrientes em um meio em constate agitação. A respeito dos compostos liberados pelas leveduras, com os métodos analíticos que utilizamos não foi possível definir diferenças nas concentrações dos compostos entre os métodos Charmat e Tradicional devido a essas diferenças na viabilidade das populações. Nesse caso, pode ser que utilizando métodos mais precisos e realizando a quantificação de polissacarídeos liberados pelas leveduras por exemplo, pode ser que seja constatando alguma diferença.

O que chama a atenção, é que existem diversos estudos focados em acelerar o processo de morte celular e liberação de compostos, tais como, a utilização de leveduras mutantes (Gonzalez et al., 2003; Nunez et al., 2005), utilização de micro-ondas, ultrassons e adição de enzimas β-glucanase (Gnoinski et al., 2021a, 2021b), uso de campo eléctrico pulsado (Dimopoulos et al., 2018; Martínez et al., 2019, 2018, 2016) entre outros (Comuzzo et al., 2017; la Gatta et al., 2016; Velázquez et al., 2016). A homogeneização contínua do vinho espumante após a fermentação pode ser uma forma barata e eficiente de aumentar a taxa de mortalidade celular e talvez outra opção enológica para melhorar e acelerar esse processo.

Da mesma forma que no Capítulo 1 da Tese, esse trabalho pode ser extrapolado para outras cepas de leveduras, pois da mesma maneira que há diferenças entre as cepas em outros tipos de morte (Orozco et al., 2012a), é provável que haja alguma variação também nesse caso. Outro fator que não foi testado é diferenças de temperatura durante a homogeneização. Pelo fato de termos realizados os experimentos em escala industrial, não foi possível utilizar essa variável. Entretanto, é provável que tenha influência nos sistemas com homogeneização, da mesma forma que há diminuição do tempo de vida cronológico das leveduras no método Tradicional com aumento da temperatura (Orozco et al., 2012b).

Sobre a comparação dos métodos Charmat e Tradicional referentes a análise sensorial e compostos voláteis no Capítulo 3, confirmamos nossa hipótese que afirmava que os métodos não possuem grandes diferenças desde que conduzidos de forma similar. Entre os estudos disponíveis na literatura, nosso experimento parece ser o único até o momento que conduziu as fermentações e o tempo de envelhecimento de maneira similar. Esse fato torna as comparações válidas tendo como variável principal o método de elaboração. Em alguns trabalhos disponíveis na literatura, onde as comparações foram feitas com espumantes comerciais (Julie A. Culbert et al., 2017; Ubeda et al., 2016), em nosso entendimento as conclusões referentes

exclusivamente aos métodos de produção perdem “força”, pois outras variáveis como por exemplo, variedades de uvas utilizadas, características dos vinhos base e tempo de envelhecimento dos espumantes vão influenciar muito nas características do produto.

Provavelmente nossos resultados terão um impacto entre os enólogos e no setor vitivinícola. Isso porque existe um pré-conceito de que os espumantes elaborados pelo método Tradicional tornam-se melhores devido ao seu processo de espumatização. Entretanto, como mostramos, essa etapa do processo não é a principal e não é um definidor de qualidade, pois, em nossos testes, os espumantes elaborados pelos dois métodos se diferenciam muito pouco, onde menos da metade dos avaliadores são capazes de distinguir as amostras.

As conclusões desse estudo podem ser usadas como marketing para as empresas que produzem espumantes pelo método Charmat longo e como consequência, conseguir que o produto agregue valor. Isso pode ser interessante para os espumantes brasileiros onde a sua maioria é feita pelo método Charmat. O tempo de acompanhamento abrangeu tempos de envelhecimentos comumente utilizados nos dois métodos, porém é possível estender esses tempos e pensar em comparações desse gênero com espumantes ainda mais envelhecidos (acima de 2 anos). Isso pode ser interessante como um projeto de longo prazo para a vitivinicultura nacional.

Os estudos que se encontram nos apêndices IV, V e VI realizados junto com o grupo de pesquisa italiano da *Fundazione Edmund Mach*, e apresentados nesse momento na forma de resumo, também são importantes para o avanço da ciência e especificamente, para o avanço da tecnologia voltada a elaboração de espumantes. No trabalho do apêndice IV mostramos que é possível modular os aromas dos espumantes *Prosecco* através da escolha do tipo de levedura e do tipo de nutrição utilizada durante a fermentação dos vinhos base. Esse trabalho pode ajudar de forma prática na tomada de decisão do enólogo, podendo chegar o mais perto possível do estilo de vinho planejado antecipadamente através dessas escolhas. No apêndice V e VI os estudos abrem novas possibilidades de escolha de variedades de uvas resistentes para elaboração de espumantes a partir de suas características aromáticas. Nossos resultados mostram que os espumantes elaborados com essas variedades de uvas resistentes possuem características aromáticas interessantes para a elaboração de espumantes. No contexto atual, onde se procura cada vez mais que os produtos agrícolas causem menos impacto no meio ambiente e sejam seguros para saúde, a utilização dessas variedades vão ao encontro da vitivinicultura do futuro em que a sustentabilidade tem um papel de protagonista.

A soma dos estudos realizados nessa Tese pode auxiliar nos avanços tecnológicos da viticultura e enologia, pode abrir portas para pesquisas aplicadas, além de auxiliar na

compreensão de fenômenos fermentativos considerados importantes dentro da enologia. Como sabemos, os vinhos espumantes brasileiros têm qualidade e possuem um grande potencial, é um mercado que vem crescendo e vai necessitar cada vez mais dos conhecimentos gerados através da ciência para continuar a se desenvolver. Nesse contexto, esperamos que a soma dos nossos trabalhos seja de valia para a ciência e para o setor vitivinícola brasileiro.

6 Conclusões

- Dependendo de suas concentrações, a presença de etanol e SO_2 no ambiente de fermentação causa um sinergismo que aumenta o estresse das leveduras, modificando a homeostase intracelular, desregulando o pH intracelular e aumentando o estresse oxidativo, levando à morte celular. Além disso, o etanol junto do dióxido de enxofre faz com que as leveduras aumentem a expressão de genes relacionados à tolerância ao sulfito na tentativa de superar seus efeitos negativos.
- Durante a segunda fermentação dos espumantes elaborados pelos métodos Charmat e Tradicional, na presença de carboidratos, a homogeneização contínua do líquido no tanque pelo método Charmat ou a permanência das garrafas estáticas com as borras concentradas no fundo no método Tradicional não tem influência aparente na segunda fermentação.
- Após a segunda fermentação, a homogeneização constante do espumante no método Charmat aumenta consideravelmente a perda de vitalidade e viabilidade das leveduras em comparação com o envelhecimento das células concentradas no fundo da garrafa no método Tradicional.
- Junto com o início desse declínio acelerado de viabilidade devido à agitação do tanque, há um aumento na quantidade de RNAm relacionado principalmente a marcadores de autofagia, indicando que ocorre uma autofagia antecipada no método Charmat.

  Em geral, apesar da morte antecipada no método Charmat, na forma em que os métodos foram conduzidos, os compostos quantificados relacionados com o envelhecimento sobre as borras não têm diferenças.
- A partir do mesmo vinho base e inóculo, com os vinhos envelhecendo o mesmo tempo sobre as borras, os espumantes elaborados pelos métodos Charmat e Tradicional são semelhantes nos aspectos sensoriais e analíticos.
- Quanto mais os espumantes envelhecem, mais difícil se torna a diferenciação sensorial dos produtos apenas pelo método utilizado na segunda fermentação.
- O método utilizado para realizar a segunda fermentação não define a qualidade dos espumantes. As diferenças encontradas nos espumantes feitos pelos métodos Charmat e Tradicional são sutis e não causam grandes mudanças capazes de modular as características gerais dos produtos.

Perspectivas para trabalhos futuros

- No futuro pretendemos realizar estudos mais aprofundados sobre a adaptação do inóculo para a realização da segunda fermentação de espumantes, pensando em alternativas práticas de superar o estresse causa pelo sinergismo entre o SO_2 e o etanol presentes no vinho base. Além disso queremos compreender se o tipo de morte que o sinergismo desencadeia é majoritariamente apoptótico ou necrótico.
- Pretendemos também realizar novos ensaios e estudar mais profundamente a relação entre a homogeneização do líquido e a queda da viabilidade, provavelmente testando variáveis como temperatura e outras cepas de leveduras por exemplo, numa tentativa de otimizar o processo. Ainda sobre esse assunto, pensamos em realizar análises mais precisas de polissacarídeos, aminoácidos, peptídeos e proteínas, verificando se existe diferenças entre os dois métodos de envelhecimento (homogêneo e estático).
- Em parceria com a *Fundazione Edmund Mach* (Itália) pretendemos dar continuidade com os trabalhos relacionados com as variedades resistentes, realizando testes com essas variedades de uvas em regiões vitivinícolas do estado do Rio Grande Sul.

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8 Apêndice

8.1 Apêndice I – Poster apresentado na Jornada do Programa de Pós-Graduação em Biotecnologia (PPGBIO), 18 de outubro de 2019.

I JORNADA DO PROGRAMA DE PÓS GRADUAÇÃO EM BIOTECNOLOGIA

Caxias do Sul, 18 de outubro de 2019

ESPUMANTES TRADICIONAIS E CHARMAT: COMPARAÇÃO ENTRE SISTEMAS

Bruno Cisilotto[1,3], Fernando J. Scariot[1], Philippe Mevel[2], Juliano Perin[2], Graciele Tomazi[2], Ana Paula L. Delamare[1], Sidnei Moura e Silva[1], Sergio Echeverrigaray[1]

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Introdução / Objetivo

A contribuição benéfica do envelhecimento de espumantes em contato com as leveduras é um consenso entre os enólogos, entretanto poucos estudos têm sido realizados visando determinar e interpretar os mecanismos celulares envolvidos nos processos que levam à morte e lise das leveduras durante esse envelhecimento. Além disso, se algum conhecimento tem sido gerado em espumantes elaborados pelo método tradicional (segunda fermentação na garrafa), não há referências relacionadas à produção em sistemas Charmat (segunda fermentação em tanques de pressão), método largamente empregado no Brasil. Neste contexto, o trabalho visa avaliar as leveduras em seu comportamento fermentativo, metabólico, autofágico/autolítico/apoptótico e acompanhar a evolução da composição dos vinhos nos dois métodos, analisando suas características físico-químicas e sensoriais ao longo da tomada de espuma (segunda fermentação) e do seu posterior envelhecimento.

Metodologia

Estão sendo elaborados espumantes na empresa Chandon do Brasil (convênio UCS - Chandon), pelos métodos tradicional e Charmat, utilizando o mesmo vinho base (uvas das variedades Chardonnay, Pinot Noir e Riesling Itálico) e inóculo (*Saccharomyces cerevisiae* EC1118). Até o presente momento estamos avaliando a segunda fermentação dos espumantes nos dois sistemas, através de um acompanhamento semanal da tomada de espuma, com análises de pressão, densidade, açúcar e álcool e uma avaliação celular, através do acompanhamento da população de leveduras, integridade da membrana celular, tamanho celular e complexidade, vitalidade celular, espécies reativas de oxigênio (ROS) e apoptose, através de citometria de fluxo.

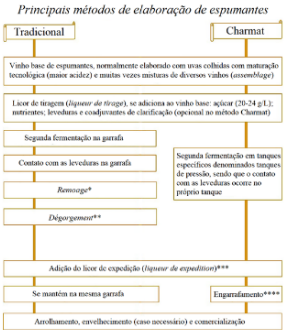

Figura 1: Diferenças e similaridades dos dois métodos mais utilizados na elaboração de espumantes com duas fermentações. * Processo pelo qual as leveduras e sedimentos (borras) contidas no vinho são movimentadas no sentido do gargalo da garrafa sendo que ao final do processo todo o sedimento está acumulado no gargalo para posterior retirada; ** Retirada dos sedimentos da garrafa, sendo que esse processo pode ser feito com o congelamento do bico da garrafa (*à la glace*) ou sem o congelamento (*à la volée*); *** O licor de expedição é basicamente composto de açúcar que irá definir o "estilo" do espumante (extra brut, brut, demi-sec e outros), ácido orgânico caso haja necessidade de correção e conservantes, sendo o dióxido de enxofre (SO_2) o mais utilizado; **** Antes do engarrafamento do espumante é realizado uma filtração isobárica. Adaptado de: (DI GIANVITO et al., 2019; TOGORES, 2018).

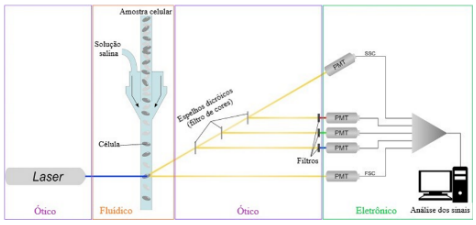

Figura 2: Citômetro de fluxo composto por sistemas fluídico, óptico e eletrônico. PMT= *photomultiplier tubes* (tubos fotomultiplicadores); Posições dos feixes de luz: SSC= *Side-scattered* (dispersão lateral da luz) e FSC= Forward scatter (dispersão frontal da luz). Adaptado de (LONGIN et al., 2017).

Resultados e Discussão

A temperatura da tomada de espuma foi controlada, variando de 11 a 13 °C e teve uma duração de aproximadamente 70 dias nos dois métodos. Através das análises de acompanhamento das fermentações e acompanhamento celular, foi possível realizar gráficos com curvas de fermentações nos dois sistemas, avaliando o comportamento individual em ambos os métodos. As avaliações celulares realizadas até o presente momento podem ser consideradas homogêneas, não sendo possível visualizar diferenças consideráveis nos parâmetros analisados entre os métodos durante a etapa fermentativa. No momento presente está sendo avaliado o comportamento celular pós-fermentativo com declínio populacional com a morte celular por ausência de fontes de carbono e diminuição das fontes de nitrogênio assimilável.

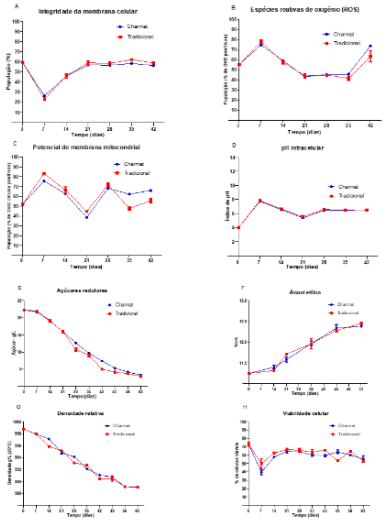

Figura 3: A, B, C e D - Citometria de fluxo e corantes específicos (equipamento FACSCalibur - Becton–Dickinson) A- *kit LIFE/DEAD* (*Thermo Fisher Scientific*, MA, EUA). B- DHE = *dihydroethidium* (corante oxidante); C- 3,3' *dihexyloxacarbo cyanine iodide* (DioC6); E- Método *Lane-Eynon* (AOAC, 1995); F - destilação eletrônica do etanol e medição da densidade (BRASIL, 1986). H- Microscópio de luz e azul de metileno.

Considerações finais

Os resultados apresentados até o momento, são a parte inicial do nosso projeto que tem previsão de acompanhamento até 500 dias. Após esse período e com todas as análises previstas realizadas (análises físico-químicas, moleculares, sensoriais e outras) esperamos obter dados suficientes para compreender um pouco mais sobre os mecanismos e as particularidades de cada método, podendo assim descrever as diferenças e similaridades e as etapas aonde elas ocorrem.

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8.2 Apêndice II – Artigo técnico referente ao capítulo 1 – Revista IVES Technical Reviews

IVES Technical Reviews vine & wine

https://doi.org/10.20870/IVES-TR.2022.5433

The synergism between SO_2 and ethanol is the "villain" of yeasts at the beginning of the second fermentation of sparkling wines

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In our paper, we show that at a pH commonly found in sparkling base wines (i.e., 2.8-3.3), a synergism occurs between the SO_2 added as a preservative and the ethanol present in the base wine. This synergism causes high stress on yeasts that lead to an important loss of viability, which in turn causes an increase in the lag phase time, slow fermentation and difficulties in finishing the *prise de mousse*.

Hypothesis creation and experimental design

The hypothesis that synergism between SO_2 and ethanol is the major stress factor at the beginning of the second fermentation of sparkling wines emerged from the observation that yeast population viability decreased and lag phases differed in length in some fermentations within the first week after *pied-de-cuve* inoculation.

As mentioned in the introduction and the discussion of our paper[1], the stress caused by ethanol and SO_2 is described in the literature. There are calculations and formulas in the oenological chemistry literature which theorise the fraction of molecular SO_2 taking into account the concentration of ethanol[2], as well as studies showing the synergism caused by SO_2 and ethanol in "finished" wines with wine spoilage yeasts. In addition, it is recommended that free SO_2 concentrations in sparkling base wines be less than 10 mg/L before starting the second fermentation[3]. However, we did not find any research in the scientific literature describing the synergism between SO_2 and ethanol and showing the stress caused in yeast strains commonly used for the second fermentation of sparkling wines.

With this in mind, we created an experimental fermentative model to test the behaviour of the yeast *Saccharomyces cerevisiae* EC-1118® (strain specifically marketed to produce sparkling wines) in different environments. The treatments comprised a synthetic medium with i) added SO_2, ii) added ethanol, iii) added SO_2 and ethanol, and iv) neither SO_2 nor ethanol added (control). In this way, it was possible to evaluate and compare the behaviour of the yeasts in each fermentative system via analyses with stress markers, cell vitality and viability markers and the expression of genes related to SO_2 stress.

Interpreting the results

The results obtained in our experiments showed that synergism between SO_2 and ethanol intensifies stress and affects the yeast population in a significant way, with a decrease in vitality and viability, increased production of of reactive oxygen species (ROS), decreased intracellular pH, increased production of acetaldehyde, and increased expression of genes related to stress caused by SO_2[1]. The same effect of decreasing yeast vitality and viability was observed in an industrially conducted second fermentation with real wine[1]. Figure 2 illustrates the effect of each treatment in terms of viability and fermentation kinetics. In this case, the experiment was conducted at a temperature of 20 °C. Cell viability was assessed with

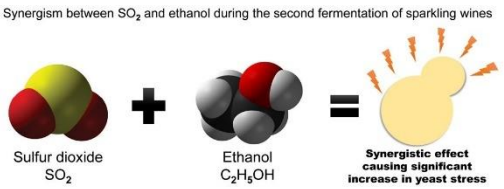

FIGURE 1. Informative graphic summary of the synergistic effect of SO_2 and ethanol.

DNA intercalating dye propidium iodide (PI) using a flow cytometer (Figure 2A) and the evaluation of reducing sugars using the method with 3,5-dinitrosalicylic acid (DNS) as reducing agent and microplate reader with absorbance at 595 nm (Figure 2B) (for more information see article). The results in Figure 2 show the behaviour of an inoculum adapted with ethanol in the different treatments, simulating the beginning of a second fermentation. In the treatments with the addition of SO_2 only, the yeasts can be seen to have very similar behaviour to the control, despite undergoing a small reduction in viability during the first hours of adaptation. In the treatment with ethanol alone, there is no loss of viability, but there is a decrease in fermentation speed and consumption of sugars due to the inhibition of growth of the population[1]. In the treatment with 5 % ethanol and 20 mg/L SO_2 it is interesting to note that, although the yeasts underwent a slightly

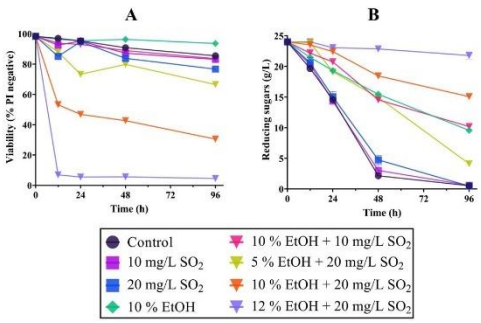

FIGURE 2. Influence of each treatment on the viability and kinetics of fermentation. Adapted from Cisilotto *et al.* (2021)[1].

more pronounced loss of viability at the beginning and suffered more stress[1], the fermentative kinetics increased speed after 48 h with an increase in the percentage of yeast viability, showing that the amount of ethanol plays an important role in the synergistic effect. In treatments with quantities equal to or above 10 % ethanol and with an initial amount of SO_2 above 10 mg/L, there was a marked decrease in the viability of the population and consequently a reduction in the speed of sugar consumption. This slows down fermentation and can eventually lead to the cessation of fermentation.

Final remarks

Despite several studies looking for a substitute for SO_2[4] for use in winemaking, one that has such a comprehensive effect (antioxidant and antimicrobial activity), as well as being low cost and safe for human consumption, does not yet seem to exist. Thus, we will probably continue to use SO_2 as a wine preservative for a long time. However, in the case of base wines used for sparkling wines, which is an intermediate product, the doses of free SO_2 must be very precise and personalised. For this precise dosage, besides the concentration of free SO_2, and the pH index, ethanol concentration, storage temperature, wine turbidity and ionic strength of each wine must be considered. The amount of yeast cells inoculated for the second fermentation of sparkling wines plays an important role in the quality of the product; an excess of cells at the time of inoculation can cause sensory defects. The lower the concentration of ethanol and the amount of free SO_2 in the base wine, the better the yeast adaptation and consequently the better the fermentation kinetics; the excess of cells at inoculation and excessive reduction in percentage of viable cells at the beginning of the process can thus be avoided. Carrying out a linear and standardised fermentation can avoid a series of logistical complications and unnecessary expenses, especially in sparkling wines made in the traditional method in which the re-inoculation of the *pied-de-cuve* implies the opening of the bottles. Therefore, balanced concentrations of SO_2 and ethanol will minimise not only yeast stress at the beginning of the second fermentation of sparkling wines but also the "stress" of the oenologist responsible for conducting this process. ■

Sources: *Sourced from the research article: "Yeast stress and death caused by the synergistic effect of ethanol and SO_2 during the second fermentation of sparkling wines" (OENO One, 2021).*

1 Cisilotto, B., Scariot, F. J., Vivian Schwarz, L., Mattos Rocha, R. K., Longaray Delamare, A. P., & Echeverrigaray, S. (2021). Yeast stress and death caused by the synergistic effect of ethanol and SO2 during the second fermentation of sparkling wines. *OENO One*, 55(4), 49–69. https://doi.org/10.20870/oeno-one.2021.55.4.4809

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8.3 Apêndice III – Artigo técnico referente ao capítulo 1 – Revista Revue des Oenologues

TECHNIQUE

Effet de synergie entre SO_2 et éthanol sur les levures au début de la prise de mousse

Impact du changement climatique sur la seconde fermentation alcoolique

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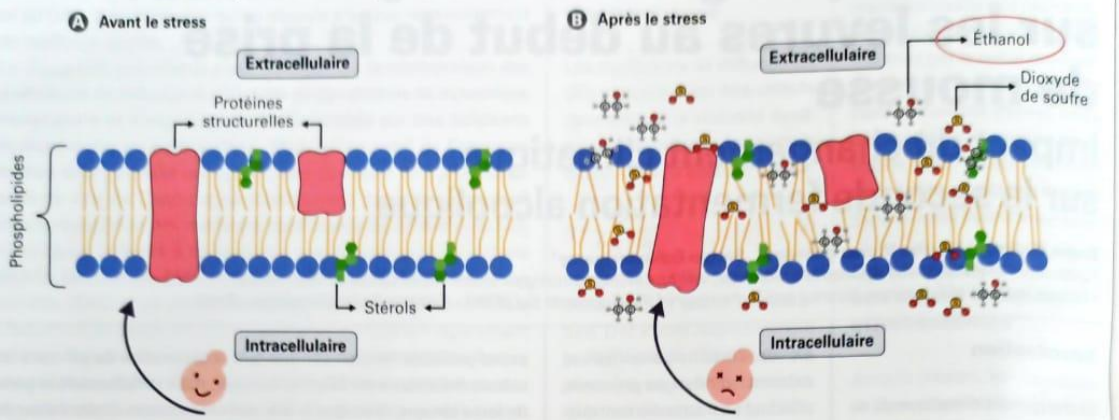
Introduction

Le changement climatique dû au réchauffement de la planète est une réalité et nous devons nous adapter. Plusieurs études dans la viticulture et dans l'œnologie montrent les effets possibles du réchauffement climatique et ses conséquences *(Schultz, 2016; Tate, 2001; Venios et al., 2020)*, ainsi que les alternatives possibles pour surmonter ces problèmes *(Gutiérrez-Gamboa et al., 2021)*. Concrètement, dans le domaine œnologique axé sur l'élaboration de vins mousseux, nous avons une aggravation technologique, car, comme nous le savons, l'un des facteurs du changement climatique est l'augmentation des températures moyennes, et cela est lié à une maturation précoce des raisins. Cela entraîne une diminution de l'acidité des moûts et des vins, ainsi qu'une augmentation du titre alcoométrique due à l'augmentation de sucres accumulé dans le fruit. Cependant, pour qu'un vin mousseux conserve ses caractéristiques de fraîcheur en plus de sa capacité de vieillissement, il est essentiel que le vin de base ait un pH relativement bas (2,8-3,3) et une acidité élevée. Il est donc probable que les conséquences du réchauffement climatique entraîneront de plus en plus d'interventions œnologiques visant à maintenir, augmenter et corriger l'acidité des moûts et des vins. Pour maintenir le pH à un niveau bas et l'acidité à un niveau élevée, certaines alternatives existent: vendanges précoces, effectuer un fractionnement qualitatif du moût pendant le pressage, ajouter des acides organiques œnologiques, empêcher le développement de la fermentation malolactique (FML) considérée comme une désacidification microbiologique, et utiliser des résines échangeuses de cations. Bien que dans les régions plus froides comme la Champagne, par exemple, le choix de réaliser ou non la FML soit une option de « style » de la maison. Dans des nombreuses autres régions du monde, l'inhibition de cette biotransformation est une nécessité technique, car la perte possible de cette acidité par conversion de l'acide malique en acide lactique par les bactéries lactiques, doit probablement être corrigée d'une manière ou d'une autre. Cependant, pour éviter l'apparition de la FML, il est nécessaire d'inhiber ou d'éliminer les bactéries lactiques. Dans ce cas, les doses de SO_2 utilisées sont relativement plus importantes. En ce qui concerne les résines échangeuses de cations, elles interagissent avec les différents cations présents dans les moûts et les vins, réduisant ainsi leur concentration *(Cisilotto et al., 2020; Ponce et al., 2018)*. Cette diminution des cations réduit le pH, mais aussi la force ionique. Cela peut augmenter la fraction de SO_2 moléculaire dans les vins *(Waterhouse et al., 2016)*, et passe inaperçues dans l'analyse de routine de la cave qui ne prend probablement en compte que la diminution du pH dans les calculs théoriques du SO_2 moléculaire, mais difficilement la perte de force ionique. Bien que le SO_2 soit un excellent conservateur du vin avec une large action antioxydante et antimicrobienne *(Lisanti et al., 2019)*, nous devons avoir un contrôle précis des doses utilisées dans les vins de base pour les vins mousseux, afin d'éviter des problèmes au début de la seconde fermentation alcoolique. Les doses de SO_2 élevées, ainsi que l'éthanol du vin provoquent une synergie qui augmente le stress des levures *(Cisilotto et al., 2021, 2022)*. En général, le degré alcoolique plus élevé des vins de base, la diminution de l'acidité et l'augmentation du pH, l'utilisation de résines échangeuses d'ions et par conséquent, l'élimination de cations (diminution de la force ionique) afin de diminuer le pH des vins, les doses de SO_2 plus élevées pour inhiber les microorganismes de la FML, peuvent également être considérées comme des conséquences directes et indirectes du réchauffement climatique. Comme nous le verrons ensuite, ces facteurs peuvent impacter la seconde fermentation alcoolique des vins mousseux.

L'effet de la synergie entre le SO_2 et l'éthanol sur la levure

Dans la prise de mousse des vins mousseux à deux fermentations alcooliques, l'étape initiale du tirage est sans aucun doute le moment crucial du processus. À ce stade, les levures responsables de la seconde fermentation seront diluées dans un nouveau milieu avec peu de nutriments et plusieurs inhibiteurs. Dans ce nouveau milieu, la phase de latence est cruciale, il est donc important d'adapter préalablement l'inoculum à ce nouveau milieu fermentaire *(Borrull et al., 2016)*. Dans une étude que nous avons récemment menée sur l'apparition de la mousse *(Cisilotto et al., 2021)*, nous avons montré le comportement d'une « levure œnologique » *(Saccharomyces cerevisiae cepa Lalvin EC-1118® – Lallemand, Canada)*, préalablement adaptée à un milieu contenant de l'éthanol, inoculée dans différents milieux synthétiques: un des milieux sans éthanol et sans SO_2, un deuxième avec seulement du SO_2, un troisième avec seulement de l'éthanol et un dernier avec les deux composés ensemble. Le milieu sans aucun inhibiteur n'a présenté aucune modification de la viabilité, avec une croissance normale. Le milieu avec SO_2 n'a connu qu'une faible diminution de la viabilité qui a été rapidement surmontée par la croissance de la population levurienne. Le milieu avec éthanol n'a connu aucune perte de viabilité, mais une diminution de la vitesse de croissance de la population, tandis que le milieu avec les deux composés a provoqué une synergie, augmentant considérablement la perte de vitalité et de viabilité de la population de levures. Dans

Figure 1 : Schéma d'interaction de l'éthanol avec la membrane plasmique de la levure et la possible facilitation de l'entrée de SO_2 dans le milieu intracellulaire, entraînant une perte d'homéostasie et par conséquent une perte de vitalité et de viabilité cellulaire.



le processus de prise de mousse, comme dans un moût *(Ochando et al., 2020)*, la levure quittera la phase de latence et commencera à se multiplier lorsque la majeure partie de la fraction de SO_2 libre plus réactive aura été combinée. Cependant, la présence d'éthanol dans le milieu de fermentation entrave ce processus, augmentant le temps de la phase de latence, ce qui difficulte la vie de la levure au début de la seconde fermentation.

Les résultats de notre étude *(Cisilotto et al., 2021)* montrent qu'en fonction des différentes concentrations d'éthanol et de SO_2 dans l'environnement de fermentation, la synergie augmente le stress de la levure, diminuant la vitalité, modifiant l'homéostasie intracellulaire, modifiant le pH intracellulaire et augmentant le stress oxydatif, conduisant par conséquent à la mort cellulaire. En outre, la présence d'éthanol et de SO_2 induit les levures à exprimer une plus grande quantité de gènes liés à la tolérance au sulfite afin de surmonter ses effets négatifs. Plus que des modifications chimiques comme le changement de pK_{a1} du SO_2 à cause de l'éthanol *(Waterhouse et al., 2016)*, nous pensons que cela est dû au fait que c'est dans la membrane plasmique de la cellule levurienne que se trouve la cible principale des effets perturbateurs de l'éthanol, et qu'il peut modifier la composition et l'intégrité de la membrane *(Tesnière, 2019)*. Dans le milieu fermentatif avec les deux composés, la diminution du pH intracellulaire, un stress oxydatif plus élevé et une plus grande quantité d'ARN messagers liés aux protéines qui servent de mécanismes de contrôle de la résistance au sulfite dans la levure, nous font penser que probablement cette modification de la membrane causée par la présence d'éthanol facilite l'entrée de SO_2 dans le cytoplasme, provoquant ainsi la perte d'homéostasie ainsi que les conséquences décrites ci-dessus. Dans la ***figure 1,*** nous montrons une interaction possible, illustrant le mécanisme d'action probable causé par la synergie entre le SO_2 et l'éthanol.

Réflexions finales

Tout indique que les conséquences du réchauffement climatique seront surmontées par les technologies développées dans la viticulture et dans l'œnologie *(Gutiérrez-Gamboa et al., 2021b, 2021a; Santos et al., 2020)*. Cependant, il est un fait que les régions traditionnelles seront les plus touchées *(Drappier et al., 2017; Reay, 2019)*, parce qu'elles ont des normes spécifiques contrôlées qui définissent une série de critères, par exemple, la délimitation des territoires et l'utilisation de cépages spécifiques. Ce fait englobe de nombreuses appellations d'origine qui produisent des vins mousseux, comme la Champagne en France, le Cava en Espagne et le Franciacorta en Italie, par exemple.

En règle générale, il est connu que dans les vins de base pour la seconde fermentation alcoolique, les concentrations idéales de SO_2 libre sont inférieures à 10 mg/L *(Alexandre, 2019)*. Toutefois, afin d'éviter que la FML ne se produise spontanément, cela n'est pas toujours possible. Un autre point à prendre en considération est que 1 % d'éthanol en plus dans le vin de base, par exemple en passant de 10,5 % à 11,5 %, peut exercer une influence sur la synergie entre le SO_2 et l'éthanol. Comme nous l'avons déjà dit, la diminution du pH avec des résines ioniques, ainsi que la diminution de la force ionique exerceront une influence sur le SO_2 moléculaire et par conséquent sur la synergie entre l'éthanol et le SO_2.

Tous ces détails techniques doivent être pris en considération avant de la prise de mousse. Cependant, il existe plusieurs possibilités et stratégies pour surmonter et prévenir ces

éventuels problèmes. Pour maintenir la teneur en alcool proche à des niveaux idéaux, il est possible par exemple, d'avancer les dates des vendanges plus précocement *(Longo et al., 2018)*, d'utiliser des différentes espèces de levures non-*Saccharomyces (Garcia et al., 2020)*, de réaliser de la nanofiltration, de l'osmose inverse, entre autres techniques *(Catarino et Mendes, 2011; Loyola Garcia et al., 2021)*. En ce qui concerne l'élimination des cations par l'utilisation de résines échangeuses, la modification de la force ionique des vins peut être mesurée par une méthode d'analyse directe (quantification des concentrations des principaux ions) ou par une corrélation avec la concentration d'un ion représentatif facile à quantifier et qui peut définir approximativement la force ionique. Pour inhiber la fermentation malolactique, on peut utiliser des adjuvants à base de lysozymes ou d'autres produits chimiques *(Lisanti et al., 2019)*, ainsi que la filtration par membrane *(El Rayess et Mietton-Peuchot, 2016)*. Le mélange de vins ayant des concentrations différentes de SO_2 avant la prise de mousse peut réduire le SO_2 libre à la fois par l'oxydation et par la dilution. Une autre stratégie intéressante serait de définir des valeurs approximatives des quantités idéales de cellules viables au moment de l'inoculation en fonction des concentrations d'éthanol et de SO_2. Les techniques d'adaptation et de nutrition de l'inoculum (pied de cuve) peuvent également exercer une influence positive et éventuellement des études connexes peuvent apporter de bons résultats applicables. Probablement, les facteurs qui peuvent aider à inhiber les modifications de la membrane dues à la présence d'éthanol et augmenter sa tolérance, comme, la composition lipidique et l'activité plus élevée de la H+ -ATPase dans la membrane plasmique *(Aguilera et al., 2006)*, peuvent aussi augmenter la résistance au SO_2 diminuant l'effet de synergie.

Bien que chimiquement l'interaction entre l'éthanol et le SO_2 soit bien décrite dans la littérature, il existe des calculs et des formules pour théoriser la fraction de SO_2 moléculaire qui tient compte de la concentration d'éthanol et de la force ionique des vins *(Waterhouse et al., 2016)*, et qu'il existait déjà des études montrant la synergie entre le SO_2 et l'éthanol dans les vins tranquilles avec des levures d'altération *(Chandra et al., 2015, 2014; Edwards et Oswald, 2018)*. Il semble que notre étude soit la première à montrer cette synergie en utilisant une souche de levure œnologique spécifiquement indiquée pour la seconde fermentation des vins mousseux. La réduction de la vitalité et de la viabilité d'une population de levures au moment de l'inoculation doit être évitée autant que possible. Une chute brutale peut empêcher ou compromettre la seconde fermentation des vins mousseux de se dérouler de manière linéaire. De plus, l'augmentation de la phase de latence à cause des concentrations de SO_2 et d'éthanol, peut entraîner des graves problèmes technologiques difficiles à surmonter. Par conséquent, comme nous l'avons conclu dans un document technique sur le même sujet *(Cisilotto et al., 2022)*, des concentrations équilibrées de SO_2 et d'éthanol minimiseront non seulement le stress des levures au début de la deuxième fermentation des vins mousseux, mais aussi le « stress » de l'œnologue chargé de conduire ce processus. ■

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NDLR** : les références bibliographiques concernant cet article sont disponibles sur le site internet de la Revue des Œnologues : **search.oeno.tm.fr

8.4 Apêndice IV – Resumo Congresso – 44th World Congress of Vine and Wine of the International Organisation of Vine and Wine (OIV) will take place from the 5th to the 9th of June Jerez de la Frontera, Cadiz, Spain.f

PO-2102

2023-3214: INTERACTION BETWEEN NUTRIENT SUPPLEMENTATION AND YEAST STRAIN ON THE AROMA OF GLERA BASE WINE FOR SPARKLING PRODUCTION

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Wine aroma of young wines is the result of complex interactions between grape variety, must composition and fermentation conditions. Diversly to other styles, the characteristic sought by winemakers of the base wines for sparkling production are linked to aroma neutrality, meaning the absence of varietal scents and a limited fruity intensity. The work aimed to study the effect of three different Saccharomyces cerevisiae strains and three commercial nutrient formulations (mineral, organic, and mineral + organic) on the concentration of the main yeast-derived aroma compounds in Glera wines from three different plots, produced at semi-industrial scale with a fermentation protocol industrially applied in prosecco-style base wine production. To this end, a quantification of the volatile compounds by gas chromatography coupled to mass spectrometry (GC-MS/MS) was conducted, and an orthonasal evaluation of wines was performed by a group of 15 experienced-winemakers of Prosecco. Among yeast strains, it was observed two different metabolic profiles: one leading to a higher fatty acid production and the corresponding ethyl esters, and other that increased the concentration of higher alcohols and acetate esters. In relation to nutrients, the addition of sole diammonium phosphate (DAP) increased the concentration of linear-chain fatty acids, ethyl esters, acetate esters and the acetylation rate, and lowered the concentration of higher alcohols respect to the sole yeast-derived amino acid formulation. The supplementation of the commercial mixture of amino acids and DAP led to undifferentiated concentrations of fatty acids, ethyl esters and produced lower acetate esters respect to the sole DAP supplementation. The orthonasal evaluation of the pleasantness showed that the mineral and the mixed nutrients supplementation tended to be preferred by oenologist, probably linked to the higher perception of the tropical and fresh fruity scents. At this regard, the yeast assimilable nitrogen supplied by each formulate was positively correlated to the amount of acetate esters produced by yeasts.

8.5 Apêndice V – Resumo Congresso – 22nd GiESCO (Group of International Experts for Cooperation on Vitivinicultural Systems) meeting at Cornell University in Ithaca, N.Y., July 17-21, 2023.

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AROMA CHARACTERISATION OF MOLD RESISTANT SPARKLING WINES PRODUCED IN A WARM-TEMPERATE AREA

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Abstract:

Context and purpose of the study - In recent years, resistant varieties have returned to the attention of the wine sector as a response to climate change and the reduction of pesticides in grapevine management, which is the main culprit of pesticide use in European agriculture. In this context, the production of sparkling wines could be strongly influenced due to its requirements for a particular balance between sugars and acidity, and the necessity of sound grapes to ensure wine quality. However, these parameters are not the only ones that define the suitability of a grape variety to produce sparkling wine. Among them, the aromatic profile of sparkling wines plays a crucial role in the perception of the quality of the final wines. Some active compounds present in sparkling wines are the consequence of the interaction between variety and environment and evolve during ageing. This work aims to study the volatile composition of traditional method sparkling wines produced from resistant varieties cultivated in an experimental plot in Trentino, comparing the results with those of Chardonnay, the main variety used in this area nowadays for this product.

Material and methods – Ten different varieties (Bronner, Solaris, Johanniter, Souvignier Gris, Palma, Charvir, Pinot Regina, V2, V10 and V11) were cultivated in an experimental plot located in Trentino (IT). The grapes were harvested for three consecutive years (2018-2020) according to the technological maturity to produce sparkling wine. The whole bunches were pressed with a yield of 50% (w/w) and, after settling, the must fermented at 18-20°C. Five months after the end of alcoholic fermentation, the *tirage* was carried out for the *prise de mousse* at 6 standard atmospheres in 0.7 L bottles, according to the traditional method for sparkling wine production. The wines were aged at 15°C until analysis after disgorgement. All vinification operations were performed under standardised conditions. Over 35 volatile compounds belonging to 6 different oenological families (acetate esters, ethyl esters, alcohols, fatty acids, terpenes and norisoprenoids) were analysed by GC-MS.

Results – Among the varieties, Souvignier Gris was characterised by methyl salicylate, 1-hexanol and n-hexyl acetate, while Solaris stood out for the concentration of β-damascone, acetate and ethyl esters. Bronner showed significant contents of some grape-derived metabolites, such as β-damascone and linalool. This terpene was also present in higher quantities in Solaris and Johanniter, while Charvir and V11 were distinguished by their higher α-terpineol content. Pinot Regina was characterised for certain amino acid-derived compounds such as 3-methylthio-1-propanol, isovaleric acid, 2-methylbutyrate and isobutyl acetate. All results were compared with those of Chardonnay sparkling wines from the same experimental plot and produced with the same protocol.

Keywords: Resistant varieties, aroma, sparkling wine.

8.6 Apêndice VI – Resumo Congresso - II International Congress on Grapevine and Wine Sciences (2ICGWS) - (Logroño, Spain) from the 8th to the 10th of November 2023.

II International Congress on Grapevine and Wine Sciences

8-10 November 2023
Logroño / La Rioja / Spain

Aroma characterization of mould resistant base wines for sparkling wine produced in a warm-temperate area at two different altitudes

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In a recent context where consumers pay increasing attention to sustainability and eco-friendly aspects in the decision-making process, the use of resistant varieties in the wine sector has returned to the attention. In this context, the use of mould-resistant grape varieties would be an opportunity for sparkling wine producers as it can reduce pesticide utilization in grape management and hence production costs.

However, the use of resistant varieties to produce the base wine may be strongly influenced due to its requirements for a particular balance between sugars and acidity to ensure the quality of the final product. In addition, the aromatic profile of base wine plays a crucial role in the perception of the quality of the sparkling wine.

This work aims to study the volatile composition of base wines produced from five resistant varieties (Bronner, Solaris, Johanniter, Souvignier Gris, Vinera) cultivated in two experimental vineyards located in Trentino (IT): one situated on the valley bottom and one in the hill. The results compared with those of Chardonnay, the main variety used in this area nowadays for this product, cultivated in the same plots. The volatiles was extracted from the base wines and the GC-MS/MS analysis allowed us to quantify the aromatic compounds belonging to different 6 chemical classes: acetates, ethyl esters, alcohols, fatty acids, terpenes, and norisoprenoids.

Among the varieties, Souvignier Gris was characterised by methyl salicylate and 1-hexanol, while Solaris stood out for the concentration of β-damascone, acetates, and ethyl esters. Bronner showed significant contents of some grape-derived metabolites, such as β-damascone and linalool. This terpene was also present in higher quantities in Solaris and Johanniter. Regarding the location, acetate esters and ethyl esters were higher in base wines of the valley bottom, and fatty acids, higher alcohols, and terpenes in the hilly plot wines.

Keywords: Resistant varieties, aroma, base wine