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Leveduras não-*Saccharomyces*: aplicação na produção de cerveja e potencial probiótico

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“Veni, Vidi, Vici. ”

Julius Caesar

Resumo

PIRAINE, Renan Eugênio Araujo. **Leveduras não-*Saccharomyces*: aplicação na produção de cerveja e potencial probiótico.** 2021. 139f. Tese (Doutorado) – Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

O mercado cervejeiro atual demanda cada vez mais novos produtos e tecnologias. Cervejas produzidas com ingredientes locais, novos *flavors*, cervejas de baixa caloria e com baixo ou nenhum teor de álcool são uma tendência do mercado. A aplicação de diferentes cepas de leveduras e bactérias comercialmente disponíveis, ou então presentes na superfície de frutas ou no interior de barris de maturação pode contribuir para obtenção de aromas únicos e características fermentativas específicas nas cervejas, e além disso, esses microrganismos podem apresentar outros atributos interessantes, como atividades que caracterizam o potencial probiótico. Cervejeiros têm buscado a utilização de leveduras não-convencionais (ou não-*Saccharomyces*) como alternativas para obter novos flavors, texturas, acidez e controle de contaminação em seus processos. Os objetivos desse trabalho foram identificar a diversidade de microrganismos presentes em cervejas de fermentação mista, com foco em leveduras não-*Saccharomyces*. Isolar e identificar leveduras não-*Saccharomyces* de fontes como flores, frutas e folhas, caracterizando-as fisiologicamente quanto características importantes para fermentação de mostos de cerveja. Avaliar o potencial probiótico das leveduras, explorando atributos como atividade antimicrobiana contra patógenos alimentares, capacidade de auto-agregação, co-agregação, tolerância às condições adversas encontradas no trato gastrointestinal e resposta induzida em macrófagos cultivados. A partir de 20 amostras de cervejas de fermentação espontânea, não-espontânea e culturas mistas iniciadoras, determinamos o metagenoma dessas amostras, compreendendo 26 espécies de fungos e 140 espécies de bactérias, observando que vários microrganismos são capazes de participar desse processo fermentativo, inclusive com presença de leveduras não-*Saccharomyces*, como *Brettanomyces, Pichia, Issatchenkia, Wickerhamomyces,* entre outras. Foram isoladas e caracterizadas leveduras não-*Saccharomyces* a partir de amostras ambientais, com destaque para *Moniliella megachiliensis*, *Pichia kluyveri, Hanseniaspora uvarum* e *Candida intermedia*, as quais apresentaram características diferentes das cepas de *S. cerevisiae* comercialmente disponíveis para produção de cerveja, como tolerância à pHs muito ácidos (pH ≤ 3.0), a elevado estresse iônico (≥ 5% NaCl), à altas temperaturas de incubação (37 °C) e a produção de aromas característicos. Foi identificada nas leveduras isoladas, a capacidade de auto-agregação em níveis superiores a 83% em diferentes temperaturas de incubação (18, 28 e 37 °C), co-agregação com *Listeria monocytogenes* e *Escherichia coli* entre 50 - 68%, inibição de crescimento de patógenos, e a imunoestimulação por transcrição de mRNAs de citocinas, fatores de transcrição e moléculas receptoras em macrófagos estimulados com células vivas das leveduras e seus derivados. Dessa forma, concluiu-se que leveduras não-*Saccharomyces* tem potencial para aplicação na produção de cervejas e potencial probiótico em condições *in vitro*, nesse caso as leveduras *M. megachiliensis*, *P. kluyveri*, *H. uvarum* e *C. intermedia*, as quais necessitam de novos estudos para determinação e confirmação da atividade probiótica e de segurança dos isolados.

Palavras-chave: fungos, fermentação, bebidas fermentadas, metagenoma, inibição de patógenos

Abstract

PIRAINE, Renan Eugênio Araujo. **Non-*Saccharomyces* yeasts: probiotic potential and applicability in beer production.** 2021. 139f. Tese (Doutorado) – Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

The current beer market constantly demands new products and technologies. Beers produced with local ingredients, new flavors, low-calorie beers with low or no alcohol content are a trend in the beer business. The application of different bacteria and yeast strains commercially available, or those present on the surface of fruits or inside maturation barrels, contribute to obtaining unique aromas and specific fermentative characteristics in beers, and in addition, other interesting attributes can be identified in these microorganisms, such as probiotic and antagonist activity against pathogens. Brewers have sought to use non-conventional (or non-*Saccharomyces*) yeasts as alternatives to obtain new flavors, texture, acidity and contamination control in their processes. Thus, the objective of this work was to identify the diversity of microorganisms present in mixed-fermentation beers, with the main focus on non-*Saccharomyces* yeasts. Moreover, isolate and identify non-*Saccharomyces* yeasts from sources such as flowers, fruits and leaves, physiologically characterizing them focusing on important characteristics for the fermentation of beer worts. In addition, evaluate the probiotic potential of the strains, exploring attributes such as antimicrobial activity agains food pathogens, auto-aggregation capacity, co-aggregation, tolerance to adverse conditions found in the gastrointestinal tract, and finally, evaluate the response induced in immune system cells when incubated with yeasts. Twenty samples of mixed starter cultures, spontaneous and non-spontaneous fermentation beers, were used to determine their metagenome, in which were identified 26 species of fungi and 140 species of bacteria, proving that several microorganisms are able to participate in this fermentation process, including a large presence of non-*Saccharomyces* yeasts, such as *Brettanomyces*, *Pichia*, *Issatchenkia*, *Wickerhamomyces*, among others. It was possible to isolate and characterize non-*Saccharomyces* yeasts from environmental samples, especially *Moniliella megachiliensis*, *Pichia kluyveri*, *Hanseniaspora uvarum*, *Candida intermedia*, which showed different characteristics from most of the commercially available *S. cerevisiae* strains for beer production, such as tolerance to very acidic pHs (pH ≤ 3.0), high ionic stress (≥ 5% NaCl), high incubation temperatures (37 °C), and the production of characteristic aromas. It was identified in these isolates an auto-aggregation capacity with levels above 83% in different incubation temperatures (18, 28 and 37 °C), co-aggregation with *Listeria monocytogenes* and *Escherichia coli* between 50 – 68%), growth inhibition of pathogens, and the induction of mRNA transcription of cytokines, transcription factors and receptor molecules by macrophages stimulated with live yeast cells and their derivatives (heat-killed cells, culture supernatant and DNA). Therefore, it was concluded that non-*Saccharomyces* yeasts showed potential for application in beer production, it being described here the yeasts *M. megachiliensis*, *P. kluyveri*, *H. uvarum* and *C. intermedia*, which also provoke interest of new studies for determination and confirmation of their probiotic activity.

Keywords: fungi, fermentation, fermented beverage, metagenome, pathogen inhibition

Sumário

1. Introdução geral

O processo de fabricação de cerveja tem seu início datado há milhares de anos, com diferentes origens geográficas sendo propostas, como Mesopotâmia, China e América do Sul, em torno de 10.000 A.C. (Callejo et al., 2020). Os antigos “cervejeiros” domesticaram leveduras para produção de cerveja devido à seleção dos melhores agentes de fermentação, ainda que o completo funcionamento do processo ainda não fosse conhecido (Lodolo et al., 2008). Somente no meio do século 17 que cientistas desvendaram e entenderam o processo fermentativo, permitindo que técnicas de isolamento, seleção e inóculos puros pudessem ser desenvolvidas e posteriormente aprimoradas (Basso et al., 2016). O processo cervejeiro está em constante evolução, e com a difusão do fenômeno microcervejeiro (ou *revolução artesanal*) nos últimos anos, há o aumento do interesse em inovação e produção de cervejas com alta complexidade sensorial (Iorizzo et al., 2021). Nos últimos 10 anos o mercado de cervejas artesanais foi impulsionado nos Estados Unidos, Europa, e principalmente, na América Latina, com grande destaque para o Brasil, o qual vivencia um crescimento em torno de 14,4% no número de microcervejarias no país, com mais de 33.000 registros de produtos no mercado (Desiderio et al., 2019; MAPA, 2021).

Tradicionalmente os métodos de produção de cerveja são divididos em duas categorias: (a) fermentação na parte inferior do fermentador, realizada pela levedura *Saccharomyces pastorianus* na produção de cervejas do tipo lager; e (b) fermentação na parte superior do fermentador, em que *S. cerevisiae* mantem-se na superfície do mosto cervejeiro, produzindo cervejas do tipo ale. Estendendo o conceito para fermentações mistas, duas novas categorias podem ser incluídas: (c) fermentações não-espontâneas, realizadas por culturas iniciadoras desenvolvidas *in-house*, as quais consistem de leveduras e bactérias ácido-láticas (BAL); e (d) fermentações espontâneas, nas quais microrganismos como enterobacterias, leveduras, fungos, BAL, bactérias produtoras de ácido acético (BAA), entre outros, são introduzidos através do ar ambiente ou fontes externas (ex: madeira, flores, frutas, etc) para fermentar essas cervejas (Vriesekoop et al. 2012; De Roos and De Vuyst 2019). Além de diversas cepas selvagens de *Saccharomyces* spp. que podem ser encontradas em fermentações mistas, outras leveduras não-convencionais (também conhecidas como não-*Saccharomyces*) podem participar da microbiota envolvida na fermentação dos mostos de cerveja (Stewart 2016; Capece et al. 2018; Molinet and Cubillos 2020).

A demanda por produtos inovadores na indústria cervejeira estimulou a busca por leveduras alternativas, principalmente não-*Saccharomyces* (Capece et al. 2018). *Brettanomyces* spp. fazem parte de um grupo de leveduras não-convencionais de aplicação mais bem-sucedida na produção de cervejas, responsáveis por contribuir com *flavors* exóticos (ex: similares a manga, abacaxi, pêra, uva), fermentação de açúcares complexos e notas acéticas em estilos específicos (ex: Belgian Lambics e *gueuzes*) (Gibson et al. 2017b; Serra Colomer et al. 2019). A experiência positiva a partir da utilização de *Brettanomyces* despertou o interesse em outras leveduras para produção de cerveja, principalmente àquelas de gêneros geralmente encontrados

em fermentações espontâneas, como *Pichia, Hanseniaspora, Candida, Lachancea, Kluyveromyces, Torulaspora, Metschnikowia,* entre outros (Steensels and Verstrepen 2014). A introdução dessas leveduras em processo cervejeiro depende de sua caracterização, aspectos de segurança, identificando a melhor maneira de aplicá-las, seja a partir de culturas puras, fermentações sequenciais ou co-fermentações (Capece et al. 2018).

A utilização de leveduras não-convencionais normalmente depende do isolamento desses microrganismos a partir de amostras do ambiente ou de fermentações espontâneas, assim há a necessidade de primeiro domesticá-los, e principalmente identificar características como a habilidade de metabolizar e fermentar açúcares, tolerância a condições encontradas durante a fermentação de cerveja (ex: etanol, pH, pressão), produção de compostos voláteis e segurança (Steensels and Verstrepen 2014; Steensels et al. 2019; Iorizzo et al. 2021). Dentre as espécies de leveduras selvagens que podem ser encontradas, algumas são ainda pouco exploradas em ambientes industriais de cervejarias, enquanto outras foram caracterizadas para outros objetivos de aplicação (Steensels et al. 2019).

Moniliella megachiliensis é uma levedura já descrita para produção de eritritol, um adoçante de grande interesse do mercado produzido naturalmente pela levedura (Inglis et al. 1992; Ghislain et al. 2002). Sua aplicação em outros bioprocessos ainda é pouco explorada, dessa forma para utilizá-la pela primeira vez na produção de cerveja demonstra-se de fundamental importância caracterizar seu comportamento em mostos formados por açúcares oriundos do malte e compostos do lúpulo. *Pichia kluyveri* é uma levedura que já foi utilizada para produção de cervejas de baixo ou nenhum teor alcoólico (Saerens and Swiegers 2017) e que além dessa, têm outras aplicações industriais sendo investigadas, como na fermentação de outras bebidas como tequila e vinho (Amaya-Delgado et al. 2013; Gutiérrez et al. 2018), biocontrole (Labbani et al. 2015; Gross et al. 2018) e quanto seu potencial probiótico (Ogunremi et al. 2015b). *Hanseniaspora uvarum* é amplamente estudada quanto sua presença em adegas e no impacto no aroma de vinhos (Grangeteau et al. 2016; Martin et al. 2018), contudo ainda é pouco explorada quanto sua aplicação na fermentação de cervejas. *Candida intermedia* pode ser considerada uma levedura pouco conhecida em todos os aspectos, porém novos estudos emergem referentes a sua atividade antimicrobiana (Acuña-Fontecilla et al. 2017; Peña et al. 2020) e capacidade de conversão de xilose (Geijer et al. 2020).

As leveduras citadas anteriormente sintetizam o potencial de leveduras não-*Saccharomyces* em aplicações industriais, tanto na produção de bebidas fermentadas, quanto no controle biológico de outras espécies. Esse controle baseia-se na atividade antagonista a outros microrganismos, entre eles patógenos alimentares, como bactérias e outros fungos (Younis et al. 2017). Essa atividade pode fazer parte de características probióticas nessas leveduras, que compreendem ainda auto-agregação, co-agregação com patógenos, tolerância às condições do trato gastrointestinal (TGI) e atividade imunoestimulatória (Hatoum et al. 2012; Ogunremi et al. 2015b; Staniszewski and Kordowska-Wiater 2021). Diversas leveduras não-*Saccharomyces* vêm sendo estudadas quanto seu potencial probiótico (Foligné et al. 2010;

França et al. 2015; Ogunremi et al. 2015b; Cassanego et al. 2017; Oliveira et al. 2017; Amorim et al. 2018; Agarbati et al. 2020), o que aumenta o número de microrganismos com possível aplicação em alimentos funcionais. Com esse propósito pesquisadores têm aliado as propriedades probióticas de leveduras e sua capacidade de fermentar bebidas, buscando desenvolver assim cervejas funcionais, com baixo teor alcoólico, alta atividade antioxidante e atributos sensoriais positivos (Canonico et al. 2021; Pereira de Paula et al. 2021; Reitenbach et al. 2021; Silva et al. 2021).

Dessa forma investigamos a diversidade de microrganismos, com foco em leveduras não-*Saccharomyces*, identificando-as em cervejas de fermentação mista produzidas de maneira espontânea e não-espontânea. Isolamos, caracterizamos e avaliamos leveduras não-*Saccharomyces* obtidas de diferentes fontes quanto sua adaptabilidade em mostos de cerveja, bem como o potencial probiótico desses isolados visando sua aplicação futura em cervejas funcionais.

2. Revisão bibliográfica

2.1. Leveduras selvagens: a importância das leveduras não-convencionais em cervejas de fermentação mista

Leveduras são ubíquas no ambiente, sendo frequentemente isoladas de fontes ricas em açúcar, como superfícies de frutas e exsudatos de plantas, no solo e em alguns insetos (Rao et al. 2008; Tikka et al. 2013). Essas leveduras, também conhecidas como leveduras selvagens, naturalmente estão presentes nos insumos utilizados para fermentação de vinhos e cervejas, como em uvas e cereais maltados, participando da microbiota que compõe suas superfícies (Molinet and Cubillos 2020). *Saccharomyces* spp. são comumente isoladas a partir de amostras ambientais, sendo abundantes e dominantes em diferentes tipos de fermentações espontâneas, o que despertou o interesse de sua seleção para aplicação em processos fermentativos (Steensels and Verstrepen 2014). A partir da sua manutenção em ambientes industriais controlados, *S. cerevisiae* e *S. pastorianus* tiveram sua atividade fermentativa caracterizada, permitindo a padronização da produção de cervejas Ale e Lagers, respectivamente (Capece et al. 2018). Baseado na história bem-sucedida de domesticação de leveduras *Saccharomyces* spp., leveduras selvagens de outros gêneros também começaram a ser exploradas em laboratórios e cervejarias quanto sua aplicabilidade de forma controlada na fermentação de mostos (Steensels and Verstrepen 2014).

Leveduras não-convencionais são alternativas interessantes para a indústria no desenvolvimento de novos produtos (Gibson et al. 2017a). Essas leveduras podem participar da fermentação de diversas bebidas, como hidromel (Barry et al. 2018) e vinho (Ciani et al. 2009), para produção de bioetanol (Ruyters et al. 2015), para produção de proteínas recombinantes (Karbalaei et al. 2020), entre outros, representando ferramentas de grande variabilidade genética com vasto potencial biotecnológico (de Souza Varize et al. 2019; Molinet and Cubillos 2020).

Alguns desses microrganismos podem ter a capacidade de naturalmente suportar condições extremas (ex: altas temperaturas e pressão), produzir metabólitos secundários espécie-específicos ou ainda apresentar tolerância a inibidores produzidos durante diferentes processos fermentativos (Navarrete e L. Martínez 2020), assim apresentando características que os tornam capazes de atuar em determinados bioprocessos sem a necessidade de engenharia genética.

O mercado envolvendo a comercialização de leveduras para diferentes fins (panificação, bebidas alcoólicas, bioetanol, entre outros) foi estimado em 2020 em um valor de 3.9 bilhões de dólares, com estimativa para 2025 de aproximadamente 6.1 bilhões (https://www.marketsandmarkets.com/). Na Europa, a indústria de leveduras cresce a uma taxa anual de 8.8% (período 2013-2018), produzindo anualmente mais de 1 milhão de toneladas desses microrganismos e exportando em torno de 30% desse total (Parapouli et al., 2020). Esse setor é quase totalmente dominado por produtos desenvolvidos com leveduras do gênero *Saccharomyces*, havendo uma limitação na oferta de leveduras não-*Saccharomyces* para o setor cervejeiro, em que somente algumas cepas de *Brettanomyces* spp. estão disponíveis como culturas iniciadoras para fermentação de cervejas (Iorizzo et al., 2021). A introdução de leveduras não-*Saccharomyces* de forma controlada para produção de cerveja ainda é recente quando comparada a indústria do vinho, para a qual já existem comercialmente disponíveis mais de 42 produtos (>79% sendo culturas puras), entre eles composições contendo leveduras como *Torulaspora delbrueckii*, *Lachancea thermotolerans* e *Metschnikowia pulcherrima* (Vejarano e Gil-Calderon, 2021). Baseado nessa aplicação, pode-se predizer que o potencial biotecnológico de novas culturas para obtenção de produtos inovadores para o mercado cervejeiro está em expansão e deve ser explorado (Iorizzo et al., 2021; Roudil et al., 2019).

A variedade de leveduras comercialmente disponível de *S. cerevisiae* e seus híbridos já não é suficiente para as indústrias vinícola e cervejeira que buscam cada vez mais produtos com novas propriedades (Molinet and Cubillos 2020). No mercado cervejeiro existe a compreensão que a produção utilizando apenas o gênero *Saccharomyces* spp. limita as características sensoriais e acaba por reduzir a complexidade do produto final (Steensels and Verstrepen 2014). *Brettanomyces* spp., *Candida* spp. e *Pichia* spp. foram historicamente consideradas contaminantes nos ambientes cervejeiros (Priest e Campbell, 1996), no entanto, atualmente são consideradas leveduras importantes na produção de cervejas de alto valor agregado, as quais apresentam novos sabores e aromas ao mercado cervejeiro (Michel et al. 2016b; De Roos and De Vuyst 2019; Tyakht et al. 2021).

Esses microrganismos fermentadores muitas vezes são utilizados apenas através de fermentações abertas (ou expostas ao ambiente), um processo muitas vezes imprevisível e que pode gerar grandes perdas econômicas às cervejarias (Lentz et al. 2014; Steensels and Verstrepen 2014). Na indústria cervejeira, mantém-se a técnica de aplicar culturas puras na fermentação, contudo observa-se que em outras indústrias, como a do vinho e de laticínios fermentados, co-culturas ou a adição de múltiplas cepas de forma controlada é uma prática comum. Essa prática possibilita a obtenção de produtos característicos com *bouquet* aromático

obtido de acordo com a concentração das cepas utilizadas no processo fermentativo (van Rijswijck et al. 2017a; Holt et al. 2018). Assim, a co-fermentação de cervejas utilizando *Saccharomyces* spp. e outras leveduras selvagens (de forma controlada e intencional) também pode agregar características distintas ao produto final na indústria cervejeira.

Em fermentações tradicionais de cervejas como Belgian Lambics e American Coolship Ales, comunidades mistas de bactérias e leveduras atuam no mosto cervejeiro, resultando em um produto único fruto da atividade de microbiotas específicas ao local de produção (Bokulich et al. 2015). A identificação das espécies presentes nessas fermentações permite determinar quais são frequentemente isoladas nesses processos, sugerindo assim que características intrínsecas ao microrganismo favorecem sua adaptação nesse ambiente, como a metabolização de açúcares complexos, tolerância ao baixo pH, produção de compostos antimicrobianos, entre outros (Rodhouse and Carbonero 2019). Nesse sentido, estudos de mapeamento de microbioma como os realizados por Spitaels et al. (2014), Bokulich et al. (2015), De Roos et al. (2019), De Roos and De Vuyst (2019), Bossaert et al. (2021) e Tyakht et al. (2021) demonstraram que dezenas de espécies de leveduras podem estar presentes em diferentes estágios da fermentação de cervejas de fermentação mista, e além de *Saccharomyces* spp., outras têm importante participação nesse processo fermentativo, como *Brettanomyces* spp., *Candida* spp., *Debaryomyces* spp., *Hanseniaspora* spp., *Kluyveromyces* spp., *Pichia* spp., *Torulaspora* spp., *Wickerhamomyces* spp., entre outras. Visto que essas podem apresentar diferenças metabólicas, como por exemplo a capacidade ou incapacidade de consumir maltose, atividade oxidativa (efeito Crabtree negativo) ou não-oxidativa (efeito Crabtree positivo) e a produção de *flavors* específicos, é de fundamental importância caracterizá-las para conhecer sua participação no processo e estabelecer o potencial para futura utilização em novas fermentações de forma controlada (De Roos and De Vuyst 2019).

2.2. Caracterização de leveduras selvagens e sua utilização para fermentação de cerveja

O isolamento de leveduras selvagens é uma prática já bem estabelecida; entretanto, o processo torna-se complexo quando há a necessidade de caracterizar os isolados antes de sua aplicação em processos fermentativos. Leveduras não-convencionais geralmente apresentam um menor rendimento na produção de etanol quando submetidas a fermentação de mostos de cervejas, por isso geralmente são aplicadas a partir de co-fermentações ou fermentações sequenciais com *Saccharomyces* spp., permitindo assim o completo consumo dos açúcares aliado a obtenção dos *flavors* produzidos por essas leveduras (Holt et al. 2018; Iattici et al. 2020). Ao passo que a produção de compostos voláteis é espécie-específico, diferentes leveduras podem resultar em fermentações com diferentes níveis de álcoois, ácidos e ésteres (van Rijswijck et al. 2017a), como observado por Saerens and Swiegers (2017), Holt et al. (2018) e Canonico et al. (2019), os quais detectaram diferentes níveis de acetato isoamilico e acetate etílico em cervejas produzidas com *P. kluyveri*, *L. thermotolerans* e *W. anomalus* associadas a *S. cerevisiae.* Nesses casos, embora em diferentes concentrações, foi possível observar que a

utilização de leveduras não-*Saccharomyces* pode potencializar a presença de *flavors* e diminuir *off-flavors* (ex.: diacetil) nas cervejas produzidas.

Fermentações espontâneas representam um nicho importante para o isolamento de leveduras selvagens, principalmente quando essas leveduras são aplicadas para fermentação com a mesma finalidade. Um dos melhores exemplos de aplicação de leveduras selvagens são as do gênero *Brettanomyces*, descritas há mais de 100 anos a partir de seu isolamento em cervejas inglesas e que resultaram no primeiro microrganismo patenteado na história, as quais são utilizadas atualmente para obtenção de *flavors* específicos, bem como no desempenho de uma atividade fermentativa particular (Steensels et al. 2015). A utilização desses microrganismos passa pela sua domesticação, a qual por definição é a sua adaptação através do tempo, por meio de uma nutrição seletiva que impacta em mudanças morfológicas e fisiológicas, a partir de um estado selvagem o qual é controlado em benefício do homem (Steensels et al. 2019). Como já realizado com *S. cerevisiae*, a domesticação se torna importante, selecionando e mantendo espécies selvagens para obter variantes capazes de se desenvolver de maneira controlada, mesmo sob condições subótimas comparadas àquelas encontradas em seu ambiente natural (Gallone et al. 2016; Gallone et al. 2018; Steensels et al. 2019; Molinet and Cubillos 2020).

Para a aplicação dessas leveduras diferentes testes devem ser executados para determinar atributos como tolerância ao álcool, habilidade de metabolizar diferentes tipos e concentrações de carboidratos, e sobrevivência em condições adversas (ex: pH, temperatura) (Tikka et al. 2013). No processo de seleção de leveduras com potencial para indústria cervejeira, é de grande importância identificar os aromas produzidos durante e após a fermentação (ex: ésteres, álcoois fúseis, fenóis), perfil de floculação e atenuação, e especialmente curvas de crescimento (Osburn et al. 2016). A partir de testes *in vitro*, centenas de isolados podem ser caracterizados fisiologicamente de forma rápida e eficaz quanto ao metabolismo de glicose, frutose, sacarose, maltose e maltotriose, os principais açúcares presentes no mosto cervejeiro (Methner et al. 2019). Além disso, métodos de *screening* de leveduras para fermentação de mostos de cerveja compreendem a caracterização da utilização de aminoácidos, o crescimento na presença de compostos do lúpulo (ex: ácidos α e β) e a tolerância a diferentes concentrações de etanol (Michel et al. 2016a). Conhecidas essas características, o potencial de aplicação dos isolados para produção de cerveja pode ser identificado. Durante a caracterização de leveduras selvagens, diferentes atributos podem ser identificados nesses microrganismos. Esses atributos podem ser interessantes para diferentes aplicações como: produção de bebidas (van Rijswijck et al. 2017b; Canonico et al. 2019), pães (Tsegaye Z et al. 2018; Bitrus et al. 2020), bioetanol (Ruyters et al. 2015), biorremediação (García-Béjar et al. 2020) e como probióticos (Fernandez-Pacheco Rodríguez et al. 2018a; Senkarcinova et al. 2019; Agarbati et al. 2020). Dentre essas aplicações, destaca-se a busca pelo potencial probiótico de leveduras, o qual vêm sendo extensivamente estudado por diversos grupos.

2.3. Atividade probiótica de leveduras

Saccharomyces cerevisiae e S. boulardii destacam-se por sua atividade probiótica. A busca por leveduras selvagens com potencial probiótico ocorre a partir de diferentes fontes, como alimentos fermentados de origem vegetal e animal, frutas, flores, insetos, madeiras, bebidas de fermentação selvagem, entre outras (Csutak et al. 2013; Zivkovic et al. 2014; Ogunremi et al. 2015a; Cassanego et al. 2017; Oliveira et al. 2017; Amorim et al. 2018). Cepas selvagens de *S. boulardii* inclusive podem ser encontradas em bebidas de fermentação espontânea, como demonstrado por Tyakht et al. (2021), que identificaram sua presença em cidras. Diferentes espécies de leveduras selvagens podem possuir potencial probiótico semelhante as do gênero *Saccharomyces* spp., dependendo da caracterização desses isolados frente a sua atividade probiótica.

Algumas leveduras possuem a habilidade de produzir compostos antimicrobianos, capazes de inibir o crescimento de bactérias patogênicas e outros fungos (Younis et al. 2017). Essa é uma das características dos microrganismos que podem conferir a denominação de "probióticos", os quais por definição são microrganismos vivos que quando administrados em quantidades adequadas conferem benefícios à saúde do hospedeiro (FAO, WHO, 2001). Para serem considerados probióticos, esses microrganismos necessitam possuir algumas propriedades funcionais como: não ser patogênico, resistir às condições do TGI, capacidade de adesão celular, ter ação imunoestimulatória, entre outras (Bevilacqua et al. 2009; Fakruddin et al. 2017). Para que sua aplicação industrial seja viável, aspectos metabólicos tais como: utilização de diferentes substratos, crescimento em diferentes temperaturas e pHs, tolerância ao estresse osmótico e atividade enzimática devem ser caracterizados nas cepas (Cassanego et al. 2017).

A possível atividade probiótica das leveduras é observada através de diferentes características, como por exemplo a atividade antagonista a bactérias e fungos patogênicos, como realizado por Younis et al. (2017) e Fakruddin et al. (2017). Leveduras probióticas são eficazes no tratamento de infecções bacterianas que resultam em diarreias (ex: diarreia do viajante), na diminuição dos efeitos inflamatórios de doenças e síndromes (ex: doença de Chron) e atualmente estudadas referente ao seu impacto positivo no sistema nervoso central (Czerucka et al. 2007; Birmann et al., 2021). A capacidade de limitar o crescimento de outros microrganismos pode estar relacionada a produção e secreção de proteínas extracelulares (Fakruddin et al. 2017), especialmente toxinas *killer* (Younis et al. 2017). Além disso, leveduras podem aderir bactérias patogênicas por meio de sua parede celular, fixando-as em sua superfície e impedindo que um número expressivo desses patógenos ligue-se a células do hospedeiro (Tiago et al. 2012), consequentemente impedindo também sua multiplicação no intestino humano (Zeng et al. 2019). Embora a atividade antimicrobiana seja um dos principais atributos pesquisados na caracterização de um microrganismo probiótico, outras características como a tolerância às condições do TGI também são de fundamental importância, pois pode haver a diminuição ou perda de funções importantes relacionadas ao efeito probiótico caso as células não estejam viáveis durante o trânsito no TGI (de Almada et al. 2016). Assim, com frequência é

avaliada a viabilidade celular após a incubação com enzimas, sais biliares, pH e temperatura que simulem o suco gástrico e pancreático, o que pode revelar a incapacidade das leveduras em manterem-se viáveis nessas condições (Cassanego et al. 2017) ou a capacidade de tolerá-las, como observado em diferentes estudos como Yildiran et al. (2019), Oliveira et al. (2017), Ogunremi et al. (2015b), entre outros.

Microrganismos no trato gastrointestinal impactam no metabolismo, sistema endócrino, sistema nervoso, desenvolvimento do TGI e regulação do sistema imune (Thomas and Versalovic 2010). O microbioma presente no TGI é essencial para o desenvolvimento e função da mucosa intestinal, representando uma barreira central na linha de defesa contra invasão de patógenos (Canny and McCormick 2008). A composição da microbiota é capaz de prevenir e tratar desordens no intestino (ex: síndrome do intestino irritável), auxiliar em desordens sistemáticas (ex: alergias) e ainda potencializar a resposta do sistema imune à vacinas (Thomas and Versalovic 2010; Roos et al. 2012; Roos et al. 2018; Gonçalves et al. 2021). Mudanças nessa microbiota podem ser obtidas por meio da administração de bactérias e leveduras probióticas, resultando por exemplo na imunomodulação da secreção de citocinas por células imunes (Smith et al. 2014).

O trato gastrointestinal está constantemente sendo exposto a substâncias exógenas (bactérias, fungos, peptídeos, partículas de alimentos, entre outros), devido a isso 60-70% das células imunes presentes no organismo estão presentes nesse ambiente (Nakata K. 2013). Macrófagos são responsáveis por iniciar a resposta contra microrganismos, fagocitando e identificando-os através de receptores específicos, apresentando antígenos para outras células imunes e participando da coordenação da resposta imune por meio da expressão de citocinas (Duque and Descoteaux 2014). Padrões moleculares associados a microrganismos (*Microbe-associated molecular patterns – MAMPs*) em leveduras são reconhecidos por receptores de reconhecimento de padrões (*Pattern recognition receptors – PRRs*) (ex: TLR2, Dectin-1, entre outros) presentes em células imunes e assim ativando ou suprimindo respostas imunes (D. Foey 2018). Componentes da parede celular de leveduras, como β-glicanos e mananas, são reconhecidos por esses receptores, induzindo respostas específicas por meio de citocinas e quimiocinas, as quais estimulam a diferenciação de células T (Levitz 2010; Bazan et al. 2018).

A imunomodulação exercida por *S. boulardii,* principalmente na polarização da resposta imune para um perfil baseado em células e citocinas anti-inflamatórias, tem diversos relatos tanto em experimentos *in vitro* quanto *in vivo*, em diferentes modelos biológicos, como ratos (Foligné et al. 2010), suínos (Wojnicki et al. 2019), peixes (Tewary and Patra 2011) e ovinos (Santos et al. 2021). Mesmo que leveduras façam parte de um mesmo grupo de microrganismos, essa é uma atividade espécie-específica, até mesmo restrito a cepas, as quais podem gerar uma potente indução de citocinas específicas ou então serem imunologicamente inertes (Smith et al. 2014). Sugere-se que diferenças em processo metabólicos, e principalmente relacionadas a estrutura da parede celular (Lozančić et al. 2021), afetam diretamente a forma de interação com

componentes do sistema imune, consequentemente seu reconhecimento e a resposta mediada por células como macrófagos e células dendríticas (Bazan et al. 2018; Navarro-Arias et al. 2019).

Nesse sentido, Smith et al. (2014) apresenta um extensivo estudo sobre a resposta de células dendríticas à diferentes leveduras, *Saccharomyces* e não-*Saccharomyces*, destacando que algumas cepas dos gêneros *Kluyveromyces*, *Debaryomyces* e *Metschnikowia* foram responsáveis por estimular a produção de níveis de citocinas (IL1β, IL6, IL10, IL12 ou TNF-α) iguais ou superiores aos observados para *S. boulardii.* A imunoestimulação realizada por leveduras também é uma característica interessante quando se objetiva sua utilização como sistema de expressão e entrega de moléculas de interesse biotecnológico (Bazan et al. 2018), visto que o reconhecimento dessas células e a resposta gerada podem influenciar na apresentação dessas moléculas. O potencial probiótico de leveduras não-*Saccharomyces* tem sido extensivamente estudado por diversos grupos (Younis et al. 2017; Amorim et al. 2018; Fernandez-Pacheco Rodríguez et al. 2018b; Zeng et al. 2019; Agarbati et al. 2020; Fernández-Pacheco et al. 2021a), entretanto dados referentes a imunomodulação exercida por esses microrganismos ainda são escassos na literatura.

2.4. Leveduras selvagens, atividade probiótica e produção de cerveja

A atividade probiótica de *S. boulardii* é conhecida há mais de 50 anos e sua capacidade de produção de cervejas vem sendo amplamente estudada (Staniszewski and Kordowska-Wiater 2021). Aliar o efeito probiótico de leveduras juntamente de suas aplicações, como a produção de cervejas de baixo ou nenhum teor alcoólico, pode ser uma opção atrativa a indústria e ao consumidor que buscam formas inovadoras de consumo desses microrganismos. Bebidas não-lácteas são interessantes como possíveis fontes de probióticos, pois satisfazem a necessidade do público vegetariano e podem ser consumidas por indivíduos intolerantes a lactose (Rošul et al. 2019; Canonico et al. 2021). Além de ser uma fonte de minerais, vitaminas, polifenóis e fibras, investiga-se a capacidade funcional de cervejas, as quais tem potencial de aplicação como bebida probiótica (quando fermentadas com microrganismos probióticos, sejam eles leveduras e/ou bactérias) (Mulero-Cerezo et al. 2019; Canonico et al. 2021). A utilização de cervejas como sistema de entrega de probióticos é um assunto atual, ainda pouco estudado, em que novos estudos emergem explorando essa alternativa (Senkarcinova et al. 2019; Canonico et al. 2021; Reitenbach et al. 2021; Silva et al. 2021).

A bioprospecção de cepas selvagens com características probióticas e sua aplicação na produção de cervejas é um tema na fronteira do conhecimento, principalmente relativo ao efeito da administração desses microrganismos via bebidas fermentadas em humanos. Enquanto isso, estudos preliminares com cervejas fermentadas por leveduras probióticas destacam os efeitos benéficos ao organismo de modelos animais, como na promoção de um comportamento anti-depressivo (Silva et al. 2021). Outros estudos como Pereira de Paula et al. (2021) relatam que

há a possibilidade de cervejas serem utilizadas como forma de entrega de probióticos pois *S. boulardii* resistiu em concentrações mínimas ideais após a produção da bebida, sua armazenagem e a passagem por simulações das condições encontradas no TGI. Leveduras são capazes de suportar essas condições após meses de armazenagem pois apresentam boa tolerância a níveis significativos de etanol, a altas concentrações de compostos do lúpulo e a diferentes temperaturas de armazenagem, bem como manutenção de reservas energéticas celulares (Senkarcinova et al. 2019; Pereira de Paula et al. 2021; Ramírez-Cota et al. 2021; Staniszewski and Kordowska-Wiater 2021). Dessa forma, trabalhos que utilizam *S. boulardii* como modelo biológico permitem projetar a aplicabilidade desses microrganismos.

Leveduras não-*Saccharomyces* com potencial probiótico também vem sendo investigadas quanto sua capacidade de produção de cervejas funcionais, como demonstrado por Canonico et al. (2021) para *Lachancea thermotolerans* e *Kazachstania unispora*, que além de serem responsáveis por cervejas de *flavors* únicos são capazes de manter a concentração de células viáveis durante o armazenamento pós-engarrafamento. Caracterizar leveduras selvagens quanto sua habilidade fermentativa, tolerância ao processo pós-fermentativo e armazenamento, segurança na utilização e atividade probiótica demonstra-se imprescindível pois as informações ainda são escassas (Capece et al. 2018; Canonico et al. 2021; Staniszewski and Kordowska-Wiater 2021). Assim, mais estudos são necessários para identificar novas leveduras com características ideais, tanto relacionadas a atividade probiótica quanto potencial para aplicação como culturas iniciadoras para produção de cerveja.

3. Hipótese e Objetivos

3.1. Hipótese

Leveduras não-convencionais (ou não-*Saccharomyces*) de interesse da indústria cervejeira podem ser obtidas e caracterizadas a partir do isolamento em seu habitat natural.

3.2. Objetivo Geral

Identificar e caracterizar novas leveduras capazes de fermentar mostos de cerveja e que apresentem características probióticas.

3.3. Objetivos específicos

- Explorar o metagenoma de cervejas de fermentação mista produzidas de forma espontânea e não-espontânea, identificando bactérias e fungos que participam desses processos fermentativos;

- Isolar e caracterizar novas cepas de leveduras não-*Saccharomyces* capazes de participar da fermentação de cervejas;

- Avaliar o potencial probiótico das leveduras isoladas, quanto a atividade contra patógenos, auto-agregação, co-agregação, tolerância às condições do trato gastrointestinal e reconhecimento por células do sistema imune.

4. Capítulos

4.1. Artigo 1 – Mixed culture metagenomics of the microbes making sour beer

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Mixed culture metagenomics of the microbes making sour beer

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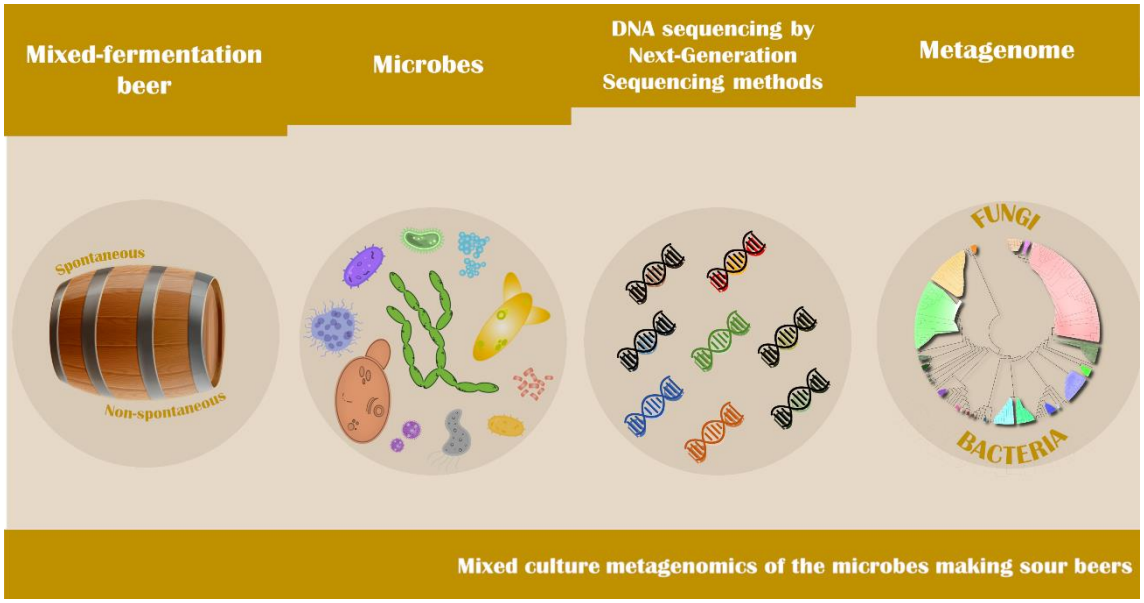
Graphical abstract



Abstract

Mixed microbial cultures create sour beers but many brewers do not know which microbes comprise their cultures. The objective of this work was to use deep sequencing to identify microorganisms in sour beers brewed by spontaneous and non-spontaneous methods. Twenty samples were received from brewers, which were processed for microbiome analysis by next generation sequencing. For bacteria, primers were used to amplify the V3-V4 region of the 16S rRNA gene; fungal DNA detection was performed using primers to amplify the entire internal transcribed spacer region. The sequencing results were then used for taxonomy assignment, sample composition, and diversity analyses, as well as nucleotide BLAST searching. We identified 60 genera and 140 species of bacteria, of which the most prevalent were *Lactobacillus acetotolerans*, *Pediococcus damnosus*, and *Ralstonia picketti/mannitolilytica*. In fungal identification, 19 genera and 26 species were found, among which the most common yeasts were *Brettanomyces bruxellensis* and *Saccharomyces cerevisiae*. In some cases, genetic material from more

than 60 microorganisms was found in a single sample. In conclusion, we were able to determine the microbiomes of various mixed cultures used to produce beer, providing useful information to better understand the sour beer fermentation process and brewing techniques.

Keywords: microbiome; mixed-fermentation; sour; beer; yeast; bacteria

Introduction

Traditionally, beer production methods are divided into two categories: (a) fermentation at the bottom of the fermenter, performed by *Saccharomyces pastorianus* in lager beerproduction, and (b) top-fermented beers, in which *S. cerevisiae* yeast ferments at the top ofthe wort, producing ales. Extending the concept to mixed fermentations, two new categories can be included: (c) non-spontaneous fermentation, carried out by an in-house starter culture which consists of yeast and lactic acid bacteria (LAB), and (d) spontaneous fermentation, in which microorganisms such as enterobacteria, yeasts, mold, LAB, and acetic acid-producing bacteria (AAB), among others, are inoculated through exposure to ambient air or external sources (e.g., wood, flowers, or fruits) to ferment these beers [1,2]. Moreover, wooden barrels and foeders used during fermentation act as additional source for microbial inoculation in beer wort [3–5].

Mixed fermentations are generally performed by yeasts and LAB in the process of creating sour beers, forming a complex microbiome that acts through their interaction and cooperation [6]. Microorganisms and their enzymes are used through biotechnological processes for acidification, alcohol production, proteolysis, lipolysis, and amino acid conversion in beer wort [1]. Various phases of fermentation can be identified in mixed fermentation beers, in which different bacteria and yeasts are isolated at specific periods [7], including novel microorganisms not yet characterized [5]. Changes in the presence and concentrations of various microorganisms suggest the existence of a microenvironment regulated according to substrate conversion and growth-limiting factors such as pH, carbohydrate concentration, oxygen, temperature, and alcohol concentration [8].

The global beer market is experiencing a resurgence in the interest in sour beers because new products and more complex flavors are being obtained by large and small production breweries around the world [4,9]. Non-spontaneous and spontaneous mixed fermentations are processes used by brewers worldwide to produce these sour beers [4]. However, most brewers do not know exactly which microorganisms are present in their mixed cultures, as well as are unaware of the relative proportions of these microbes in their starter cultures. Even though wild yeasts, LAB, and some Gram-positive bacteria are considered contaminants in the vast majority of beer fermentations, these same microorganisms are often highly desired for the production of specific sour and wild beer styles. As an example, Lambics and American Coolship Ales are beverages with unique flavor profiles generated by “spoilage” organisms [10], in which dozens of volatile compounds can be identified as a direct result of the microbial interactions and release of fermentation by-products [11]. Thus, it is of interest to know the microorganisms present in these fermentative processes, seeking to characterize the microbial diversity and parameters that

influence sensory perceptions, and to deepen the knowledge of mixed fermentation beers.The objective of this study was to identify bacteria and yeasts present in different beers and mixed-culture samples from several locations produced by homebrewers and craft breweries using spontaneous and non-spontaneous fermentation methods.

Material and methods

Samples

The samples used in this study were obtained through the crowdfunding project "Mixed culture metagenomics of the microbes making sour beer" (DOI 10.18258/13495) hosted on the Experiment platform (www.experiment.com). Each brewer collected and sent their own sample in glass beer bottles or plastic vials containing beer or slurry, which were stored at 4 °C until their analysis.

During July–December 2019, 20 samples were received, including spontaneous and non-spontaneous fermentation beers produced by homebrewers and craft brewers, as well as some samples from house cultures propagated to ferment these beers. Samples were obtained from different regions including countries such as Canada, the United States, and Israel. Furthermore, these samples had different maintenance times, ranging from a few months of storage to more than 5 years of use and propagation by the brewer. Non-spontaneous fermentation samples originated from commercial blends, bottle dregs, or cultures that were propagated and maintained by brewers, of which the exact microbial composition was not known. Spontaneous fermentation samples mostly originated through the process of exposing the beer wort to the ambient air using open fermenters. In some of these samples, it was observed that the brewers inoculated the beer using an external source that likely contained microorganisms such as wood, flowers, or fruits. Details concerning the individual samples can be found in Table 1 and Supplementary Information File S1.

Table 1. Identification and characteristics of samples of the mixed cultures received for the study.

Sample	Origin	Material	Spontaneous Fermentation	Fruits, Woods, Flowers, or Another Microbe Source Added	Culture Maintenance Time	Commercial Strains Inoculated
1	Jerusalem (IL*)	Culture pre-pitch	YES	NO	6-12 months	-
2	Alberta (CA)	Beer/slurry	NO	YES	1-6 months	New World Saison (Escarpment Labs) Brett 'M' (Escarpment Labs)
3	Alberta (CA)	Beer	YES	YES	6-12 months	-
4	Alberta (CA)	Beer	YES	NO	6-12 months	-
5	Alberta (CA)	Beer	YES	YES	6-12 months	-

6	Alberta (CA)	Beer	YES	YES	6-12 months	-
7	Alberta (CA)	Beer	YES	YES	6-12 months	-
8	Alberta (CA)	Beer	YES	YES	6-12 months	-
9	Washington (US)	Culture pre-pitch	YES	NO	2-3 years	-
10	Washington (US)	Culture pre-pitch	YES	NO	6-12 months	-
11	Ohio (US)	Beer/slurry	NO	YES	6-12 months	Sour Solera (Bootleg Biology) Mélange (The Yeast Bay) BugCounty (East Coast Yeast) Dregs from beer bottles
12	Nevada (US)	Beer	NO	YES	6-12 months	Dregs from beer bottles
13	Nevada (US)	Beer	NO	YES	6-12 months	Dregs from beer bottles
14	California (US)	Beer	NO	NO	6-12 months	In-house culture
15	California (US)	Culture pre-pitch	YES	NO	5-6 years	-
16	California (US)	Beer	NO	NO	4-5 years	In-house culture
17	California (US)	Beer	YES	NO	1-6 months	-
18	California (US)	Beer	NO	NO	2-3 years	WY3763 (Wyeast) WY3711 (Wyeast) WLP650 (White Labs)
19	California (US)	Beer	NO	NO	1-2 years	WLP565 (White Labs) Dregs from beer bottles
20	Michigan (US)	Beer	YES	NO	1-6 months	CBC-1 (Lallemand) for bottling

*IL, Israel; CA, Canada; and US, United States.

In the case of beer samples, 50 mL was processed by centrifugation at 2000× *g* for 10 min at 4 °C and the supernatant was decanted. For mixed-culture samples, a small volume(≤5 mL) was resuspended in 50 mL sterile water and treated in the same manner. Then, the pelleted cells and debris were resuspended in 500 µL of 2× DNA/RNA Shield (Zymo Research (Irvine, CA, USA) and stored at −20 °C. Samples were subsequently submitted for microbiome analysis through the ZymoBIOMICS® Targeted Sequencing Service for Microbiome Analysis by the Zymo Research company.

DNA Extraction and Sequencing

DNA extraction was performed using a ZymoBIOMICS® -96 MagBead DNA kit (Zymo Research, Irvine, CA, USA) or ZymoBIOMICS® DNA Miniprep kit (Zymo Research, Irvine, CA) with an automated extraction platform (www.zymoresearch.com/pages/microbiome-analysis-services). Bacterial and fungal identifications were performed with 10% PhiX spike-in using next generation sequencing (NGS) on an Illumina® MiSeq™ system with a v3 reagent kit (600 cycles). First, targeted libraries were

prepared for both groups. For bacteria, 16S ribosomal RNA gene-targeted sequencing was performed using a Quick-16S™ NGS Library Prep Kit (Zymo Research, Irvine, CA, USA), in which 16S primers were used to amplify the V3/V4 region of the 16S rRNA gene, maintaining good coverage and high sensitivity. Fungal internal transcribed spacer (ITS)-targeted gene sequencing was performed using the same kit described above,though ITS2 primers were used instead 16S primers, which amplifies the entire ITS region and allowes for the molecular phylogenetic sequence identification for many fungi.

PCR reactions were performed to prepare the sequencing library, controlling cycles and limiting PCR chimera formation. The quantification of final PCR products was performed with qPCR fluorescence readings and DNAs were pooled together based on equal molarity. The cleaning and quantification of final pooled libraries was performed using Select-a-Size DNA Clean & Concentrator™ (Zymo Research, Irvine, CA, USA), TapeStation® (Agilent Technologies, Santa Clara, CA, USA), and Qubit® (Thermo Fisher Scientific, Waltham, WA, USA) reagents. Negative controls (blanks) were used during all processes, as well as the ZymoBIOMICS® Microbial Community Standard (Zymo Research, Irvine, CA, USA) as a positive control, which mimics a mixed microbial community of a well-defined composition, containing Gram-negative and Gram-positive bacteria and yeasts. Additional information can be found at the ZymoBiomics™ Service website.

Bioinformatic Analyses

Sequencing results were used for taxonomy assignment, sample composition visualization, and alpha and beta-diversity analyses. Unique amplicon sequence variants (ASVs) were inferred from raw reads using the DADA2 pipeline [12]. The ASVs of bacteria and fungi were used to create three-dimensional principle component analysis (PCoA) plots using the matrix of paired-wise distances between samples, calculated by the Bray–Curtis dissimilarity. Uclust from Qiime v. 1.9.1 [13] was used for the taxonomy assignment using the Zymo Research Database, a 16S and ITS database that was internally designed and curated, as a reference. Results were re-analyzed by amplified sequence alignment using the nucleotide collection database from NCBI (National Center for Biotechnology Information, Rockville, MD) and the nucleotide BLAST tool. Taxonomy nomenclature and classification were analyzed using the Taxonomy Browser tool on the NCBI website (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser) [14].

Sequencing results were used to separately analyze the phylogenetic relationships between groups of bacteria and fungi with MEGA v.10.1.7 software for alignment, construction, and visualization of phylogenetic trees. Sequence alignment was performed using ClustalW, which was also used to construct neighbor-joining (N-J) phylogenetic trees with 1000 bootstrap trials. Circular trees were used as templates for final figures, which had schemes and colors added using Gimp v.2.10.18 software. The microbial composition for each sample was evaluated using GraphPad Prism v.7 software, which was also used to plot the data. Samples were also analyzed for taxonomy, visualization, and interactive reporting using the Knomics-Biota system [15].

Results

Based on the different pipelines used by ZymoResearch and Knomics-Biota, with ASVs examined through the analysis of each amplified fragment using the NCBI nucleotide database, we were able to determine the metagenomes of 20 different beer samples and starter cultures that originated from mixed fermentations made by both spontaneous and non-spontaneous methods.

Bacteria – 16S rRNA V3-V4 Region Analysis

Bacterial Composition

The analysis of the bacteria present in the various samples revealed 60 genera and 120 different species, with some samples containing only one species of bacteria (e.g., samples 15, 16, 17, and 18) and others containing >50 species (e.g., sample 1) (Figure 1). Based on ASV analysis, the most prevalent bacteria were *Lactobacillus acetotolerans*, which was identified in 60% of the analyzed samples (n = 12), followed by *Pediococcus damnosus* and *Ralstonia picketii/mannitolilytica*, both identified in 35% of the samples (n = 7). Although a large number of bacteria were identified at the species level, we observed that several ASVs did not allow for the differentiation of species belonging to the same genus, leading to the designation of two different species for the same ASV, such as for *L. collinoides/paracollinoids*. Only six ASVs could not be identified at the genus level, allowing only for their classification at the level of phylum, order, or family, as in the case of Myxococales bacteria classification. The microbial composition and raw reads of each sample are available in detail in Supplementary Information File S1, while the total number of bacterial and fungal species identified is shown in Figure 2.

The significant presence of bacteria from the Enterobacteriaceae family was observed in different samples, which could be >15% of the ASV total composition, as in samples 1 and 14, and could even surpass 90% of the bacterial composition, as in samples 6, 7, and 20 (Figure A1, Appendix A). However, even though many of these beers were made by spontaneous fermentation and were inoculated with external sources of microorganisms,the presence of this bacterial group was <2% of the total composition of ASVs identified in most samples (n = 14).

Figure 1. Bacterial composition of the 20 samples analyzed. Bacterial identification was performed from ASVs originating from NGS using the V3/V4 region of the 16S rRNA gene. Due to the large number of species found, colors for identification were designated according to genera, with the exception of the Enterobacteriaceae family which is identified in the graph with the red color only.

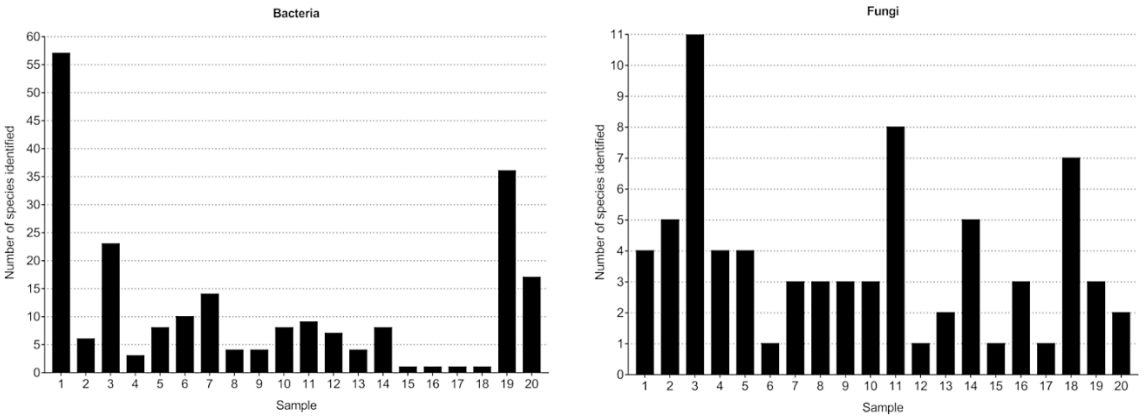
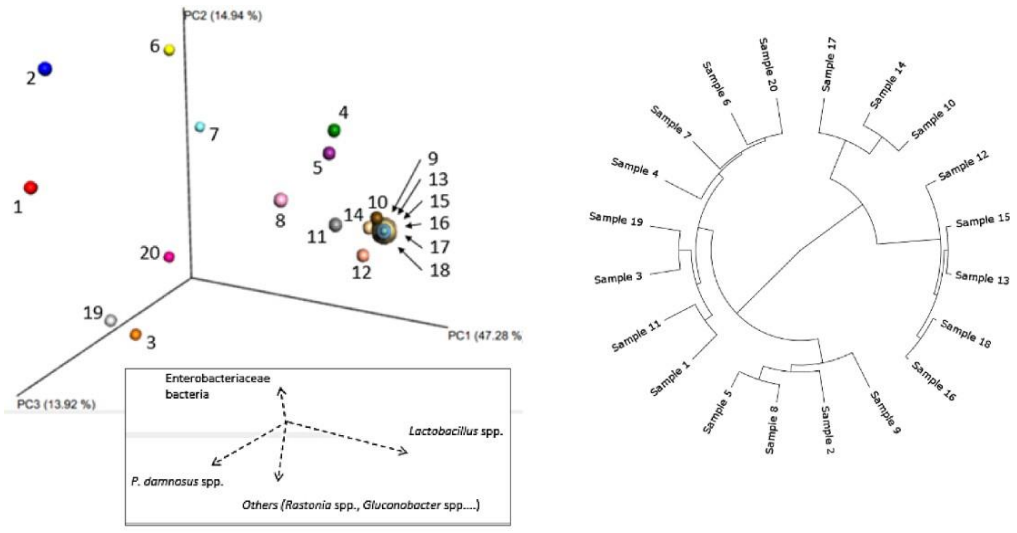


Figure 2. Number of species identified in each sample for bacteria (left) and fungi (right). Metagenome identification of mixed-fermentation samples made it possible to identify samples containing from just one to >55 species of bacteria, while for fungi, it was possible to detect samples with only one yeast participating in the fermentation and up to more than eight different species acting together during fermentation.

Based on the differences in the microbial composition among samples, beta diversity demonstrated through PCoA plots revealed some similarities among samples according to the identified genera (Figure 3a). We highlight four main groups: group A (samples 4, 5, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, and 18), in which the genus with the highest prevalence was *Lactobacillus*; group B (samples 6, 7, and 20), in which samples were composed mainly by Enterobacteriaceae bacteria; group C (samples 1 and 2), in which *Pediococcus* (*Pediococcus damnosus* exclusively) was the genus with the highest proportion; and group D (samples 3 and 19), in which the microbial composition revealed a large presence of other ASVs detected, such as *Gluconobacter* spp. and *Ralstonia* spp. Similarities observed in the bacterial composition could also be seen in the phylogenetic tree generated through the Knomics-Biota pipeline, which grouped samples in a similar way (Figure 3b). Note that the similarity between samples 1 and 11 was due to specific ASVs of *P. damnosus*, present only in these two samples.



(a) (b)

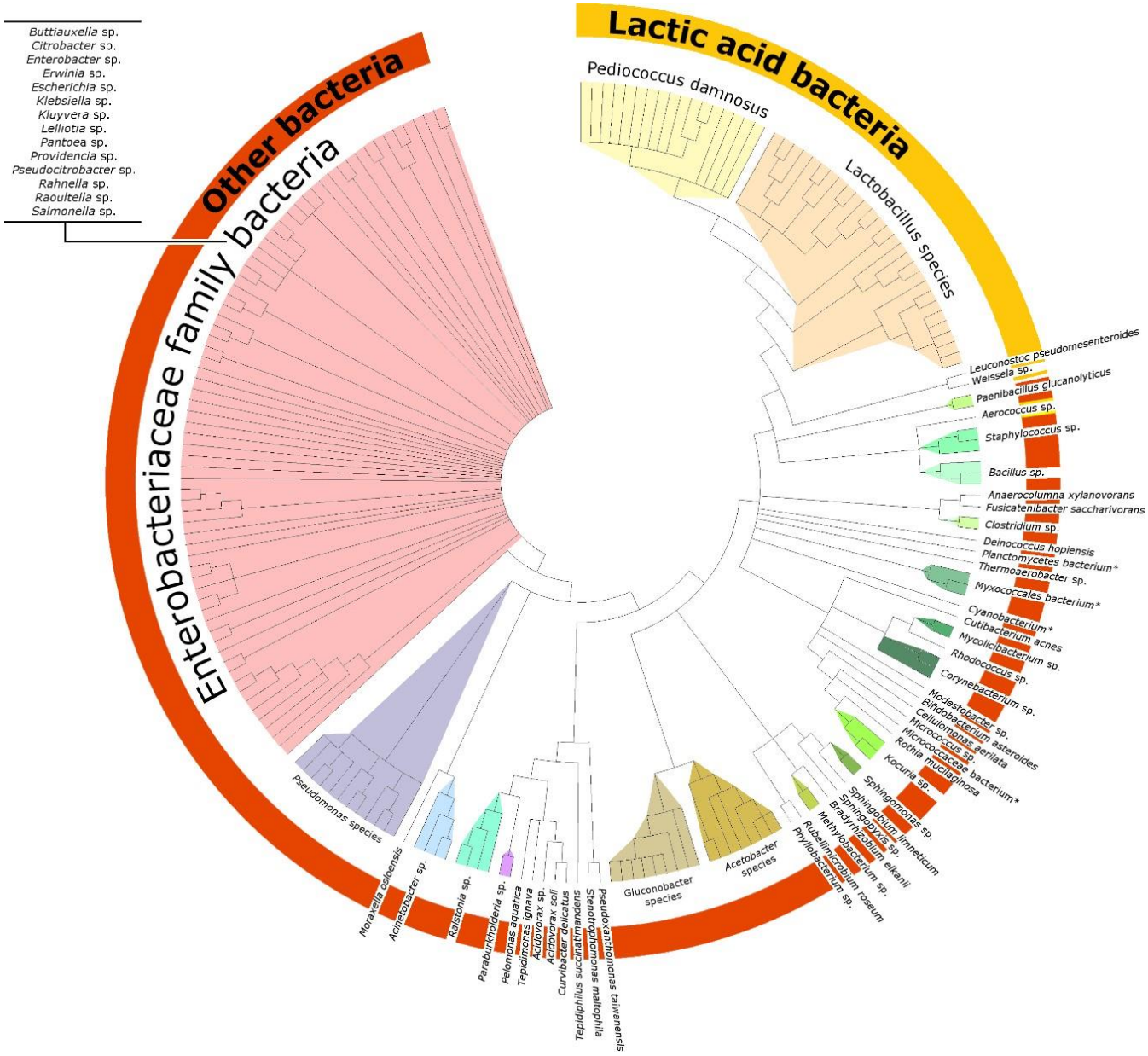
Figure 3. Sample similarity based on bacterial composition. (**a**) PCoA tridimensional plot created using the matrix of pairwise distances between samples calculated by the Bray–Curtis dissimilarity using genera found in the microbial composition. 3D images, schematic representation, and visualization were generated using the EMPeror tool. Below the PCoA plot is a schematic representation based on the sample composition, in which arrows show different directions for locating the samples on the graph according to the presence and concentration of Enterobacteriaceae bacteria, *Lactobacillus* spp., *P. damnosus*, and other bacteria such as *Ralstonia* spp. and *Gluconobacter* spp. (**b**) Phylogenetic tree showing clustering of the samples by similarity of their taxonomic composition, calculated by the Bray–Curtis dissimilarity at the ASV level using Ward's method.

Phylogenetic Analysis

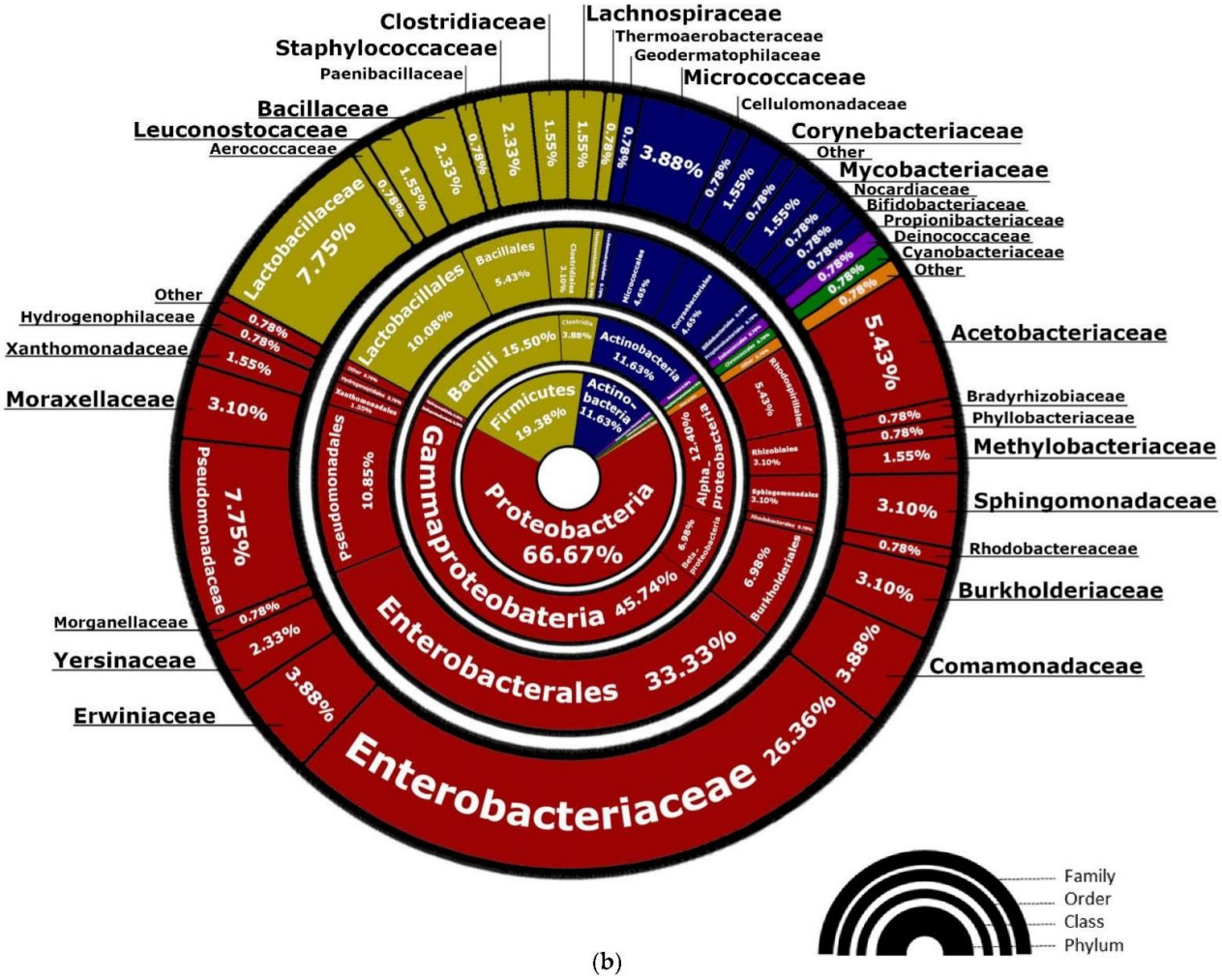
The construction of phylogenetic trees (Figure 4a) showed that *Lactobacillus* spp. and *Pediococcus* spp. were the main microbes from the LAB, a group of great importance in the production of sour beers. At least nine different *Lactobacillus* spp. were detected, including *L. brevis, L. plantarum, L. casei/paracasei, L. backii, L. buchneri, L. lindneri*, and *L. delbrueckii*, in addition to those mentioned above. Among the several species of *Pediococcus*, only *P. damnosus* was identified but the presence of possible subspecies could be responsible for the multiple different ASVs found. In addition, belonging to the LAB group, bacteria in the genera *Leuconostoc*, *Weissela*, and *Aerococcus* were likewise detected (with lower prevalence), as well as bacteria with potential probiotic activities, such as *Bifidobacterium* spp. and *Bacillus* spp. Other phylogenetically distinct groups of bacteria also demonstrated important participation in spontaneous and non-spontaneous fermentations, such as AAB *Gluconobacter* spp. and *Acetobacter* spp., and especially *G. oxydans* and *A. pasteurianus.*

Several species of the genus *Pseudomonas* were also detected, mainly in sample 20, in which it was possible to identify eight different ASVs related to this genus, corresponding mainly to *P. fluorescens*. Although *Pseudomonas* spp. are mainly known because of the pathogenic bacterium *P. aeruginosa*, this organism was not detected in the studied samples. A special emphasis was given to the Enterobacteriaceae family in the phylogenetic tree as they corresponded to a large number of identified ASVs and thus represented a vast number of species found at different concentrations. The samples' metagenomes showed the presence of a large number of genera included in this family, among them *Enterobacter* spp., *Salmonella* spp., *Klebsiella* spp., and *Pantoea* spp. During the identification of the ASVs corresponding to these bacteria, it was possible to observe a high similarity among the genera, which made specific identification difficult in many cases, especially at the species level.

In analyzing the metagenomes discovered (Figure 4b), three main phyla were identified with high prevalence: Proteobacteria (66.67% of the identified bacteria belonged to this phylum), Firmicutes (19.38%), and Actinobacteria (11.63%). ASVs from other phyla were also detected, though at smaller proportions: Deinococcus-Thermus (0.78%), Cyanobacteria (0.78%), and Planctomycetes (0.78%). Almost half of the identified bacteria belonged to the Gammaproteobacteria class, which includes families such as Enterobacteriaceae, Pseudomonadaceae, and Acetobacteriaceae, among others, totaling to 17 different bacterial families. Though most studies and applications involving mixed-fermentation beers focus on the Lactobacillaceae family, it was related to only 7.75% of ASVs, representing a small portion of the variety of microorganisms identified.


Other bacteria
Enterobacteriaceae family bacteria
Buttiauxella sp.
Citrobacter sp.
Enterobacter sp.
Erwinia sp.
Escherichia sp.
Klebsiella sp.
Kluyvera sp.
Lelliotia sp.
Pantoea sp.
Providencia sp.
Pseudocitrobacter sp.
Rahnella sp.
Raoultella sp.
Salmonella sp.
Lactic acid bacteria
Pediococcus damnosus
Lactobacillus species
Leuconostoc pseudomesenteroides
Weissela sp.
Paenibacillus glucanolyticus
Aerococcus sp.
Staphylococcus sp.
Bacillus sp.
Anaerocolumna xylanovorans
Fusicatenibacter saccharivorans
Clostridium sp.
Deinococcus hopiensis
Planctomycetes bacterium*
Thermoaerobacter sp.
Myxococcales bacterium*
Cyanobacterium*
Cutibacterium acnes
Mycolicibacterium sp.
Rhodococcus sp.
Corynebacterium sp.
Modestobacter sp.
Bifidobacterium asteroides
Cellulomonas aerilata
Micrococcus sp.
Micrococcaceae bacterium*
Rothia mucilaginosa
Kocuria sp.
Sphingomonas sp.
Sphingobium limneticum
Sphingobium elkanii
Sphingopyxis sp.
Bradyrhizobium sp.
Methylobacterium sp.
Rubellimicrobium roseum
Phyllobacterium sp.
Acetobacter species
Gluconobacter species
Pseudoxanthomonas taiwanensis
Stenotrophomonas maltophilia
Tepidiphilus succinatimandens
Curvibacter delicatus
Acidovorax soli
Acidovorax sp.
Tepidimonas ignava
Pelomonas aquatica
Paraburkholderia sp.
Ralstonia sp.
Acinetobacter sp.
Moraxella osloensis
Pseudomonas species

(**a**)



(**b**)

Figure 4. Phylogenetic analysis and abundance of bacterial taxa**.** (**a**) Phylogenetic tree constructed based on the ASVs of 120 bacterial species found in the mixed-culture samples. Phylogenetic relationships were made using MEGA v.10.1.7 software for alignment, construction, and visualization of the phylogenetic tree. Sequence alignment was performed using ClustalW. Supplementary Information File S2 contains a high-resolution version of Figure 4a, suitable for zooming and enlargement. (**b**) The image shows the prevalence of taxonomic classifications according to the genera and species found in the bacterial microbiomes. Higher taxonomic classifications are shown in circles near the image center, while lower classifications are shown toward the outer edge. Images were constructed using MEGA and GIMP 2.1 software.

Fungi

Fungal Composition

NGS targeting the ITS2 region was able to detect 19 genera and 26 different species of fungi in the 20 analyzed samples (Figure 2). Among them, yeasts were the most identified microorganisms, while ASVs from filamentous fungi, molds, and more complex fungi(e.g., mushrooms) were also identified in smaller proportions. Samples were basically dominated by two main yeasts: *Brettanomyces bruxellensis* and *S. cerevisiae*, which were present in 75% (n = 15) and 65% (n = 13) of the samples, respectively (Figure 5). Microbial composition analyses revealed that in the vast majority samples (n = 16), fermentation

was carried out by at least two different species of fungi. Of these two, an association between *Brettanomyces* spp. and *Saccharomyces* spp. was shown to be recurrent (n = 7), in which both exceeded at least 5% concentration in the ASVs found in each sample.

Other yeasts such as *Issatchenkia orientalis* (also known as *Pichia kludriavzevii*, *Candida krusei*, and *Candida glycerinogenes*) and *Wickerhamomyces anomalus* (or *Pichia anomala*) also participated in these mixed-fermentation beers, which could be detected in 30% (n = 6) and 25% (n = 5) of the samples, respectively, with a large presence in samples 3, 6, 7, and 10. It is interesting to note that not only were yeasts identified but also more complex fungi, such as *Penicillium* spp. which accounted for most of the ASVs, were found in sample 14. Overall, we observed that several fungal species were often present in mixed-culture samples, with more than seven species found in samples 11 and 18, and over 11 different fungi detected in sample 3.

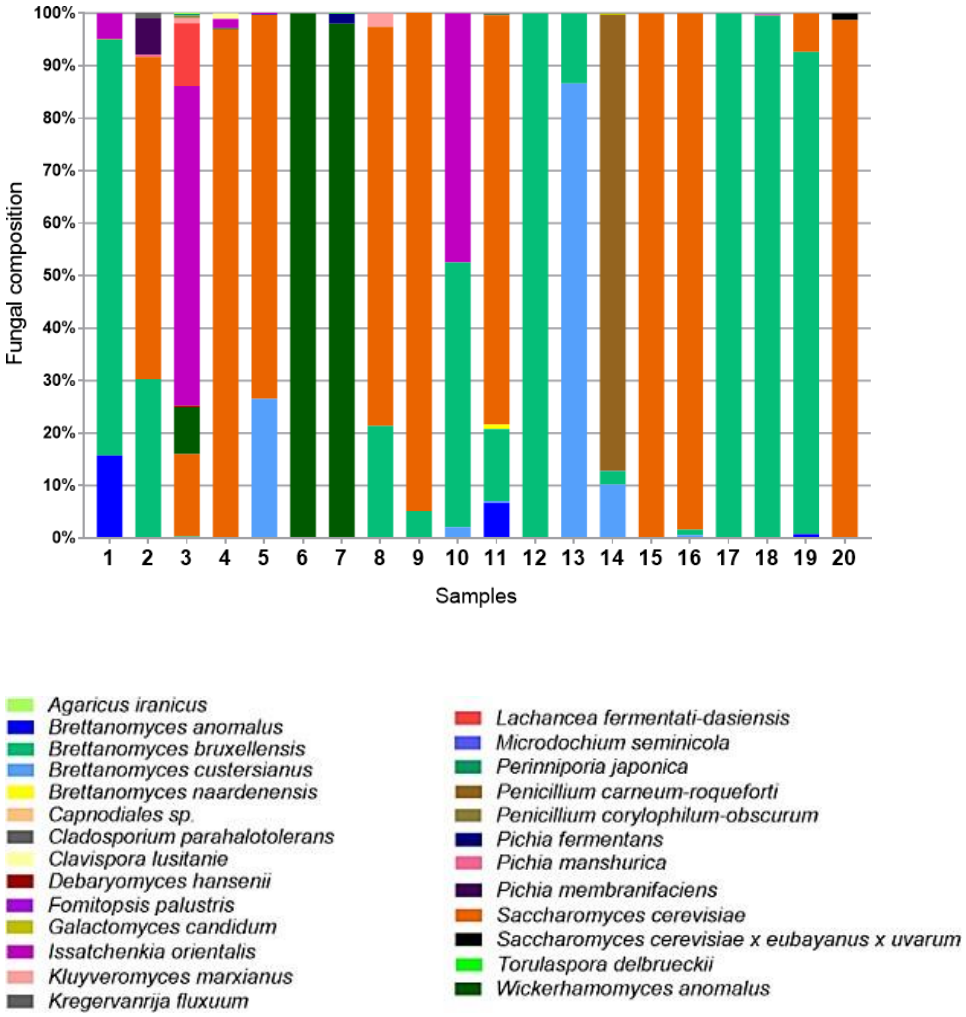
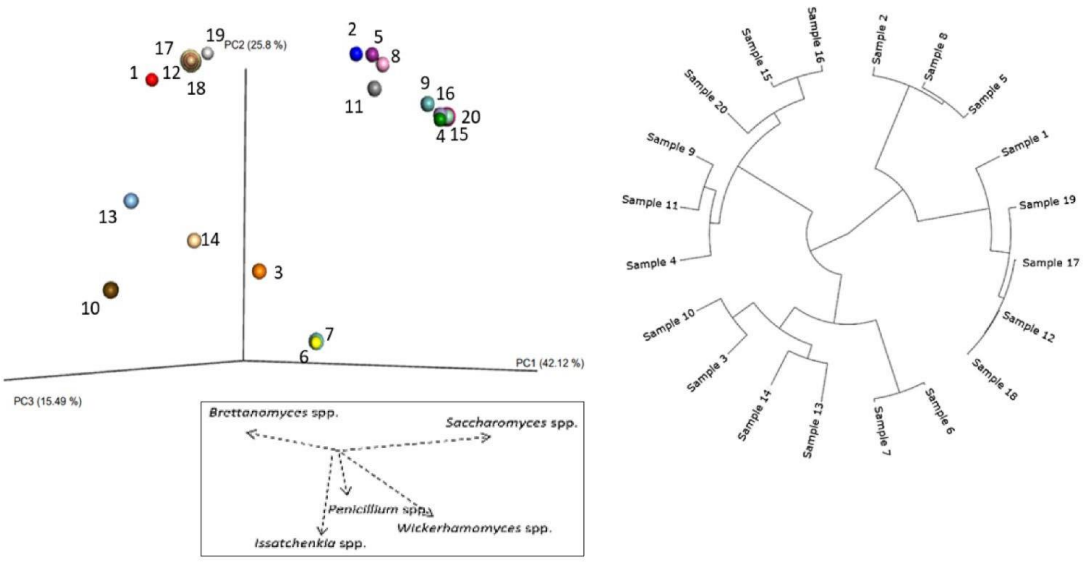


Figure 5. Fungal composition of the 20 samples analyzed. Fungal identification was performed from ASVs originating from NGS of the ITS2 region, amplifying the entire ITS region. Colors for identification were designed according to the fungal species as shown below the plot.

The beta diversity of the cultures was evaluated according to the genera identified in the microbial composition of each sample. In these analyses, we could verify the distribution of samples into four groups (Figure 6a) by similarities between identified microorganisms: group A (samples 4, 9, 15, 16, and 20), in which > 95% of the sample was composed of *S. cerevisiae*; group B (samples 1, 12, 13, 17, 18, and 19), in which > 90% of the identified ASVs corresponded to *Brettanomyces* spp.; group C (samples 2, 5, 8, and 11), in which approximately 70% of the sample was represented by *S. cerevisiae*, 25% by *Brettanomyces* spp., and 5% by other fungi; and group D (samples 3, 6, 7, 10, and 14), in which other genera such as *Wickerhamomyces*, *Issatchenkia*, *Penicillium*, and *Lachancea* dominated.

The tree constructed based on the hierarchical clustering between ASVs detected for ITS2 (Figure 6b) reveals that samples tended to follow the same relationship observed in Figure 6a, though some samples such as 13 and 14 had greater hierarchical approximation because similar ASVs referring to *Brettanomyces custersianus* were found in significant proportions (86% and 10% of the ASVs identified in these samples, respectively). Similarly, samples 9 and 11, though organized in different groups in Figure 6a, presented a phylogenetic relationship in this analysis because there were specific ASVs corresponding to *B. bruxellensis* and *B. anomalus* found exclusively in their microbial composition. The other samples displayed phylogenetic relationships in accordance with those observed in the PCoA analysis, confirming similarities in the fungi composition among the samples evaluated in this study.



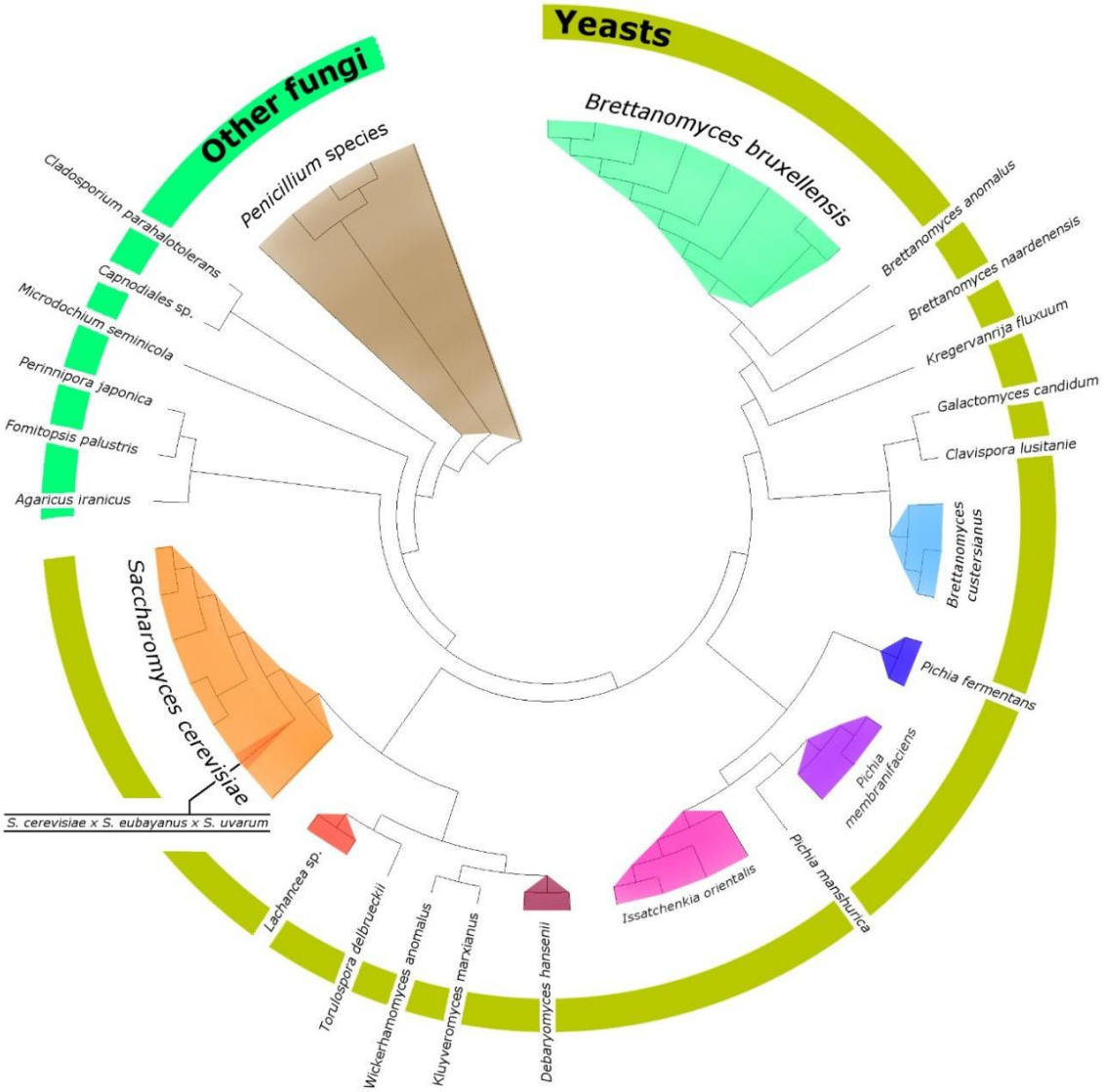
(**a**) (**b**)

Figure 6. Sample similarity based on fungal composition. (**a**) PCoA tridimensional plot created using the matrix of pairwise distances between samples calculated by the Bray–Curtis dissimilarity using the genera found in the microbial composition. Below the PCoA plot is a schematic representation based on the sample composition, in which arrows demonstrate different directions for sample localization according to the presence and concentration of *Brettanomyces* spp., *Saccharomyces* spp., *Penicillium* spp., *Issatchenkia* spp., and *Wickerhamomyces* spp. (**b**) The tree shows clustering of the samples by similarity of their taxonomic composition, calculated by the Bray–Curtis dissimilarity at the ASV level using Ward's method.

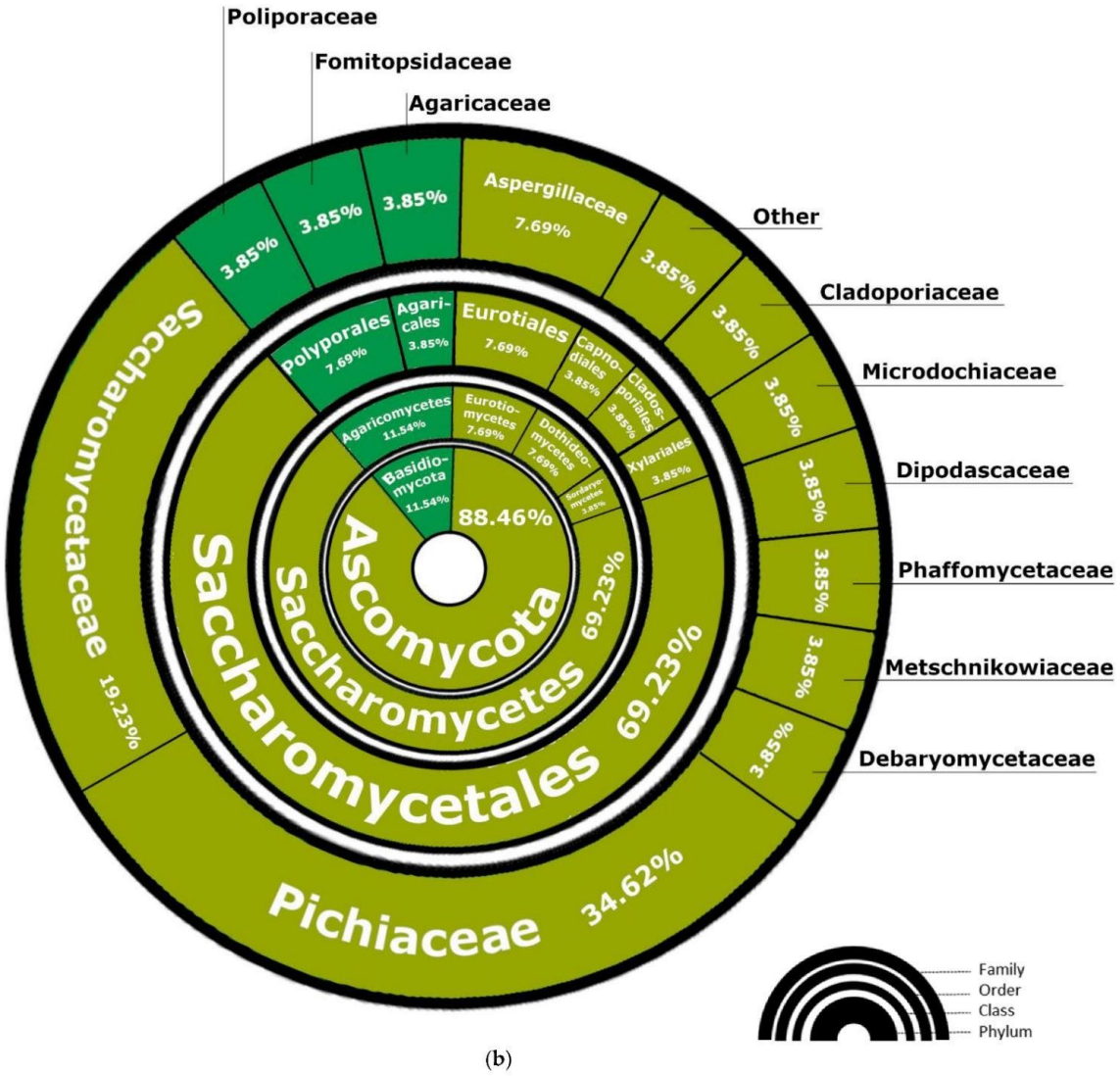
Phylogenetic Analysis

The construction of a phylogenetic tree using the fungal species detected in this study reveals different subspecies of both *B. bruxellensis* and *S. cerevisiae* (Figure 7a). Their excellent growth and adaptability in beer wort favors the presence of these yeasts, as well as the presence of other *Brettanomyces* spp. such as *B. anomalus*, *B. naardenensis*, and *B. custersianus*, all of which were identified in the spontaneous and non-spontaneous fermentation beers. In addition, multiple species from the *Pichia* genus were detected, such as *P. fermentans* and *P. membranifaciens*, with an emphasis on four subspecies of *I. orientalis* that were identified as a large proportion in some samples. We observed that different ASVs referring to the fungus *Penicillium* spp. were present in these samples, as well as more complex fungi such as *Perenniporia japonica* and *Fomitopsis palustres* (ASVs identified only in samples 3 and 18, respectively), whose presence is generally related to wood colonization.

Fungi from the Ascomycota phylum, which contains yeasts, accounted for 88.46% of the ASVs found, as well as the Basidiomycota phylum to a lesser extent (11.54%) (Figure 7b). The Pichiaceae family, which contains yeasts of the genera *Brettanomyces*, *Pichia*, *Kregervanrija*, and *Issatchenkia*, displayed the highest proportion among the families classified (34.62%) in this study. The *Saccharomyces*, *Torulaspora*, *Lachancea*, and *Kluyveromyces* genera belong to the Saccharomycetaceae family, a taxonomic classification that comprises genera of great importance in mixed-fermentation beer production, and such microbes were responsible for 19.23% of the fungi ASVs classified. Other families were responsible for the classification of only one genus, representing 3.85% each of the total yeasts found.


Other fungi
Yeasts
Penicillium species
Brettanomyces bruxellensis
Cladosporium parahalotolerans
Capnodiales sp.
Microdochium seminicola
Perinnipora japonica
Fomitopsis palustris
Agaricus iranicus
Brettanomyces anomalus
Brettanomyces naardenensis
Kregervanrija fluxuum
Galactomyces candidum
Clavispora lusitanie
Brettanomyces custersianus
Pichia fermentans
Pichia membranifaciens
Pichia manshurica
Issatchenkia orientalis
Saccharomyces cerevisiae
S. cerevisiae x S. eubayanus x S. uvarum
Lachancea sp.
Torulospora delbrueckii
Wickerhamomyces anomalus
Kluyveromyces marxianus
Debaryomyces hansenii

(**a**)



(**b**)

Figure 7. Phylogenetic analysis and abundance of fungal taxa. (**a**) Phylogenetic tree constructed based on the ASVs of 26 fungal species found in the mixed-fermentation beer samples. Supplementary Information File S3 contains a high-resolution version of Figure 7a, suitable for zooming and enlargement. (**b**) The image shows the prevalence of taxonomic classifications according to genera and species found in the fungal microbiomes. Higher taxonomic classifications are shown in circles near the image center, while lower classifications are shown toward the outer edge.

Discussion

Exploring the Microbiomes of Mixed-Fermentation Beers

Elucidating the microbiomes of mixed-fermentation beers is important to understand the participation of microorganisms in this fermentation niche. The production of sour beers from spontaneous fermentations is associated with inconsistencies in product quality, unpredictability in fermentation results, and extra time to consumption [8], as it is often not known which microorganisms will act in the beer wort exposed to the thousands of bacteria and fungi present in the environment or after the repitching a slurry from pre vious mixed-fermentation batches. Even brewers who work with spontaneously fermented beers for years or decades very often do not know which wild microbes are fermenting their beers, thus it is important to reveal the microbiomes of mixed cultures responsible for fermenting normal

beer wort into high-value sour beers. Thus, identifying and characterizing the metagenomes present in mixed fermentation beers and mixed cultures samples allows one to predict which microorganisms typically participate in these fermentations and formulate new bacterial and yeast blends to mimic this fermentation process in a controlled and reproducible way.

Preliminary studies performed by our research group concerning the metagenomes of commercial beers made by mixed fermentation and the barrels used in their maturation corroborate the data presented here, where *L. acetotolerans*, *L. brevis*, *L. buchneri*, *B. bruxellensis*, and *S. cerevisiae* were commonly observed in the samples (data not shown). Though different microorganisms have been detected at lower concentrations, the metagenome data in this study are in accordance with observations by other researchers, such as Bokulich et al. (2012) [10], Bokulich et al. (2015) [16], De Roos et al. (2019) [3], and Tyakhtet al. (2021) [15]. We observed in our work that dozens of bacterial species can be found through NGS using the V3/V4 region of the 16S rRNA, revealing that beers produced spontaneously and non-spontaneously represent an interesting source for the identification of new microorganisms that cooperate or compete amongst themselves in the consumption of substrates present in beer wort [17]. Their presence is important in mixed- fermentation beers as they are able to enhance the production of specific flavors and acidify the wort through lactic acid production, which are typical characteristics in these beerstyles [2].

Fungal ASV detection by sequencing the ITS2 region revealed microbiomes based ontwo main genera: *Brettanomyces* spp. and *Saccharomyces* spp., which were found in the vastmajority of samples corresponding to > 90% of the ASVs identified per sample. However,there is still a little-known world of unconventional yeasts to be discovered and characterized, which is comprised of less common genera such as *Issatchenkia* and *Wickerhamomyces*. The fungal microbiomes presented a lower variability in the number of species when compared to bacteria, which may be related to the avidity in glucose consumption by commercial strains (reducing the concentration of easily assimilated carbohydrates in the medium) and the competition among yeasts, which can present killer characteristics, producing secondary metabolites that aim to stop the multiplication of cross-feed competitors [18,19]. Even so, 12 different yeast genera were detected as participating in these fermentations.

Lactobacillus spp. and *Pediococcus* spp. are often identified in mixed-fermentation beers

In recent years, the application of LAB has been explored in sour beer production, with emphasis on bacteria in the *Lactobacillus* genus as not just contaminants in beer but as interesting tools for acidification and the production of new flavors [20]. Although the vast majority of these bacteria are not tolerant to hop alpha-acid concentrations of up to 20 international bitterness units (IBUs), some species and subspecies adapt to the adverse conditions that exist in beers, becoming tolerant to this and others selective pressures such as hydrostatic pressure, alcohol, and low pH [21]. In our study, we observed nine *Lactobacillus* spp., including *L. acetotolerans* (with eight identified subspecies), *L. backii*, and *L. plantarum*, a genus present in 18 of the 20 samples analyzed. Several authors highlight the presence of

Lactobacillus spp. in the microbiomes of mixed-fermentation beers, such as Tyakht et al. (2021) [15] and Spitaels et al. (2014) [7], who detected these bacteria in American Coolship Ales, Wild Ales, Sour Ales, and Belgian Lambic Beers.

P. damnosus was the only identified species from the *Pediococcus* genus but 18 subspecies were detected in this study. Similar to *Lactobacilllus* spp., *Pediococcus* spp. are commonly considered as beer and manufacturing environmental contaminants, a characteristic that encourages research into the identification of resistance factors mainly acquired through specific plasmids and genes [17,22]. In Lambic-style beers (produced by spontaneous fermentation), *P. damnosus* is one of the main isolated species and can be easily identified as part of the microbiome of the interior surface of wooden barrels [1], in the air, and on other brewery equipment surfaces [23] mainly due to its high oxidative stress resistance and hop tolerance [4]. In our study, ASVs corresponding to these bacteria were found in seven samples, representing up to 70% of the bacterial microbiome, as in sample2.

Biological acidification during the brewing process by the action *Lactobacillus* spp. or*Pediococcus* spp. has several benefits: flavor stability, greater zinc bioavailability, fast final attenuation, lower wort viscosity, and smoother hop bitterness, among others [2]. In pre-fermentation tests using these bacteria, impacts are observed on volatile compound and organic acid production, which result in significant differences in the sensorial characteristics of sour beers [20]. These bacteria can also use carbohydrates that are not metabolized by conventional yeasts, such as maltotriose, maltotetraose, and cellobiose, resulting in beer over-attenuation [8,24]. Although *Pediococcus* spp. are historically reported as responsible for high levels of diacetyl production and causing viscous “sick beers” (through exopolysaccharide secretion), these bacteria have been intentionally combined with *Brettanomyces* spp. to generate a deeper acidity and mouthfeel in various mixed-fermentation beers [24]. Some *Brettanomyces* strains have β-glucosidase enzyme activity, permitting the yeast to degrade the exopolysaccharides produced by LAB and demonstrating the importance of microbial consortia found in mixed-fermentation beers [4].

Bacteria of the Enterobacteriaceae family and their presence in spontaneous and non-spontaneous fermentations

Enterobacteriaceae bacteria are well-known contaminants in spontaneously fermented beers, participating in the initial stages of fermentation (also known as the Enterobacteriaceae phase) that starts on day one and can last approximately 1 month [1]. In our work, ASVs corresponding to different bacteria in this family were identified, such as *Rahnella* spp., *Klebsiella* spp., *Enterobacter* spp., *Providencia* spp., *Escherichia* spp., *Erwiniae* spp.,and *Pantoea* spp. In samples 6, 7, and 20, we found that almost the entire bacterial microbiome corresponded to Enterobacteriaceae, with emphasis on the species *R. aquatilis*, *K. oxytoca*, *E. asburiae*, and *E. billingiae*, which were present at levels of >20% of the ASVs found in these samples. Using methods for the isolation of microorganisms in specific culture media, Spitaels et al. (2014) [7] could verify that after 2 months of maturation, only *P. damnosus* was found in beers in which

Enterobacteriaceae were previously recovered during the initial fermentation period. Thus, it is noteworthy that although we detected ASVs corresponding to these bacteria in the samples in our study, this does not imply that these microbes were viable at the time of sample collection.

Different studies have successfully isolated and identified Enterobacteriaceae in spontaneously fermented beers, whether American Coolship Ales, Belgian Lambics, or Wild Ales [7,9,15]. In such cases, these bacteria were highly abundant (>8% of ASVs) in six beer samples. Although they are responsible for off-flavor production and can be harmful to health through biogenic amine production [1,9], these bacteria are also linked to the production of specific flavors related to young Lambics, such as 2,3-butanediol, ethyl acetate, higher alcohols, acetic acid, lactic acid, succinic acid, and fatty acids [10,24]. Manual wort acidification prior to fermentation is a technique that may lower the Enterobacteriaceae concentration during early fermentation and is often used by lambic brewers to shorten the Enterobacteriaceae phase [4].

Other bacteria

The metagenomes of our mixed-culture samples were not restricted to bacteria from the Enterobacteriaceae and Lactobacillaceae families. Several different species were often present, mainly AAB from the genera *Gluconobacter* and *Acetobacter*. Indeed, *G. oxydans* corresponded to 64.23% of the ASVs found in sample 3 and different *Acetobacter* spp. were present in concentrations of >20% in samples 2, 4, and 5. Their presence is likely due to the fact that they are able to tolerate hop alpha-acids and ethanol concentrations up to 10%, as well as being responsible for ethanol oxidation with the formation of organic acids, mainly acetic acid (one of the flavors commonly associated with spontaneously fermented beers) [25].

Similar to our findings, Tyakht et al. (2021) detected the presence of the Leuconostocacaceae and Acetobactereaceae families at abundant levels (>5%) in the microbiomes of two wild beers. Not only are LAB important in the flavor bouquet construction of sour beers, but other less conventional bacteria can also be highlighted, such as *Acetobacter* spp.which are related to the production of beers with high contents of 5-methyl-furfural, flavonoids, and 2- and 3-methyl butanol [9]. When associated with the presence of lactic acid,acetic acid addition by these bacteria (at adequate amounts) can increase the sensory complexity of the beer, resulting in the construction of the "layered flavors" so important in traditional Belgian sour beers [4,24]. However, excessive production of acetic acid and acetoin by AAB is normally avoided through the use of full and well-sealed wooden casks.This maintains a yeast pellicle at the wort/air surface that enables microaerobic conditions, consequently limiting AAB growth [4].

Bacteria of the genus *Pseudomonas* were also detected. Even though a large variability in the of ASVs for *Pseudomonas* spp. was found only in sample 20, *P. fluorescens* was identified in 20% of the studied samples (n = 4), with concentrations ranging from 0.3–8% in samples in which it was identified (samples 1, 3, 12, and 19). This genus was also identifiedby Rodhouse (2017) [26], mainly in malt samples, with a

decrease in its detection during the brewing process (mashing, boiling, and bottling). Considering spontaneously fermented beers are usually exposed to the environment, it is possible that these bacteria are transported from the raw brewing ingredients (mainly malt) to the wort during the open-air fermentation process [27].

Aside from *Pseudomonas* spp., other Gram-negative bacteria found in significant concentrations were *Ralstonia* spp., representing 17% of bacterial ASVs found in sample 19 and 8% in sample 3. Both genera were also detected at small concentrations ($<10^3$ cell/mL) by other researchers such as Takahashi et al. (2015) [28] who observed the presence of these bacteria in the early and intermediate stages of beer-like beverage fermentation and Bokulich et al. (2012) [10] who detected them in American Coolship Ales. Other bacteria such as *Acinetobacter* spp., *Stenotrophomonas* spp., *Sphingomonas* spp., and *Staphylococcus* spp. were also detected in small amounts in the metagenomes of mixed-fermentation beers by Bossaert et al. (2021) [9]. However, De Roos et al. (2019) [3] detected a high relative abundance of the same bacteria on the surfaces of wooden barrels used in beer fermentation.

Fungi besides the *Brettanomyces* spp. and *Saccharomyces* spp.

In addition to traditional and well-known *Brettanomyces* spp. and *Saccharomyces* spp., other yeasts were present in the microbiomes of our mixed-culture samples. Genera such as *Debaryomyces*, *Lachancea*, and *Pichia*, and mainly *Issatchenkia* and *Wickerhamomyces*, were identified as part of the samples' fungal microbiomes. *I. orientalis*, a yeast known for its industrial application in bioethanol production and participation in the construction of the aroma bouquet in wines [29], has been used in co-fermentations with *S. cerevisiae* for the production of beers with higher levels of fruity esters [30]. In our study, we identified this yeast at high concentrations (>45% of the ASVs found) in the fungal microbiomes of samples 3 and 14, demonstrating its adaptability to beer wort and its participation in the fermentation process.

Wickerhamomyces anomalus and *D. hansenii* are yeasts very often detected in the air surrounding coolships, as well as observed participating in Lambic beer fermentation [4].The use of *W. anomalus* has already been studied in a controlled way in the production of beers, classifying this yeast as a potential organism for the primary souring technique with contribution to fruity organoleptic profiles [31,32]. *W. anomalus* was detected in our study,comprising almost the entire fungal microbiome (>98%) in samples 6 and 7, as well as being relatively abundant in sample 3 (9% of the ASVs). Although Tyakht et al. (2021) [15] did not detect the presence of *W. anomalus* in the beer samples analyzed, Spitaels et al. (2014) [7] confirmed its participation in Lambic beer fermentation, identifying relevant levels (>20%) in the microbiomes of beers after 24 months and detecting its presence both in the air of the brewery environment and on the external surface of casks.

Varied *Pichia* spp. such as *P. manshurica* and *P. fermentans* were also found in the analyzed samples, generally at concentrations of < 2%. However, *P. membranifaciens* was the only species of this

genus that could be identified above this rate in the microbiome of a sample, representing 7% of the ASVs found in sample 2. Although in our study the concentration of *Pichia* spp. was considered low, other studies such as Bossaert et al. (2021) [9] identified *P. membranifaciens* as comprising up to 98% of the OTUs (operational taxonomic units) found in beers at specific maturation periods and also revealing that beer maturation over time leads to changes in the microbiome. *Lachancea* spp. are also reported to participate in mixed-fermentation beers, mainly being responsible for pH decrease in sour beers through lactic acid production [31,33]. *L. fermentati/dasiensis* was detected in our work in sample 3, with a relatively abundant concentration of 12% of the fungal microbiome.

The beer microbiomes in our study also revealed that among the most abundant families of fungi, Aspergillaceae represented around 7% of the identified ASVs. Cason et al. (2020) [34] observed a similar relative abundance (6.7% of OTUs) in Sesotho beer samples, a traditional South African style produced by spontaneous fermentation. Fungi of the *Penicilllium* genus, which is part of this family, are related to gushing and their presence has been detected inside beer and wine barrels, mainly participating in biofilm formation on porous surfaces [3,35]. These fungi are capable of producing enzymes responsible for the degradation of lignocellulose and betaglycans, contributing to changes in beer color and aroma [36]. Other researchers such as Bossaert et al. (2021) [9] and De Roos et al. (2019) [3] have also identified *Penicillium* spp. in their samples. However, we found only one sample (sample 14) with >80% of fungal ASVs related to *Penicillium carneum/roqueforti*. ASVs referring to fungi with advanced and more complex structures (e.g., *P. japonica*) were also detected in samples at levels between 0.05% and 0.5%, indicating little relevance in the microbiomes of the analyzed beers.

The importance of traditional yeasts in mixed-fermentation beers

Brettanomyces (also known as *Dekkera*), perhaps the second most important genus of yeast in mixed-fermentation beers, can be found in large proportions in spontaneously and non-spontaneously fermented beers, associated with the production of phenolic and esterified volatile compounds [8]. The ability of *Brettanomyces* ssp. to metabolize complex sugars, produce acetic acid, generate a characteristic aromatic profile, and cause "super attenuation" in beer wort has aroused interest in their use [32,37], which has led to several production laboratories around the world distributing these yeasts commercially.

Here, *B. bruxellensis* was the species with highest proportions found in the ASVs of this genus, totaling to >90% of the fungal microbiome in some samples such as 12, 17, and 19. Sobel et al. (2017) [38] also highlighted the large presence of *B. bruxellensis* in traditional beers from countries such as Belgium, Italy, and Switzerland, detecting it in the metagenomes of 36 samples (n = 39). Not only did we find *B. bruxellensis* in large proportions in our samples but also *B. anomalus* (15% of the ASVs in sample 1) and *B. custersianus*, which comprised >85% of the ASVs in sample 13. These species present differences in sugar metabolism, aggregation, and flavor production, leading to interest in their use during beer fermentation [39]. It is noteworthy that the ASVs corresponding to *Brettanomyces* spp. and

Saccharomyces spp. found in samples 2, 11, and 18 are possibly not related to wildstrains but rather to the use of commercial blends inoculated at the beginning of fermentation.

The identification of the yeasts in the metagenomes of our samples revealed only one species in the *Saccharomyces* genus, *S. cerevisiae*, which together with *S. pasteurianus* are the brewers' yeasts most used for beer fermentation and are widely commercially available through propagation laboratories [40]. Wild strains of *S. cerevisiae* can offer interesting characteristics such as the extracellular production of different secondary metabolites (related to the production of new aromas) and tolerance to different stress conditions (e.g., salinity, temperature, and high levels of ethanol) [19]. These strains have mainly been isolated and characterized from spontaneous fermentations of traditional fermented beverages such as Kveik strains, isolated from Norwegian Kveik Beer [41]. In seven samples, in which there was no commercial *S. cerevisiae* inoculum, ASVs corresponding to *S. cerevisiae* were detected, which may be related to the presence of wild strains acting during fermentation. However, because the detection of this yeast may be related to the cross-contamination of utensils and fermenters by the presence of commercial *S. cerevisiae* on such equipment [16], further studies must be conducted to confirm these ASVs as originating from wild strains.

Among the eight strains of *S. cerevisiae* detected in the metagenomes here, only one was identified as a hybrid of this yeast with another species (*S. cerevisiae* × *S. eubayanus* × *S. uvarum*). It was found at a low concentration (<2% of the ASVs) in just one sample (#20), a beer produced through spontaneous fermentation. Yeasts with hybrid genotypes are generally reported in beer and wine, where their presence is caused by interspecific hybridization during the diversification and adaptation of yeasts to the industrial niche [42].

Conclusion

The microbiome analysis of 20 samples of mixed cultures with different origins allowed us to deepen our knowledge of the metagenomes of the cultures used for beer production, identifying the distribution and concentration of bacteria and fungi in these samples. Exploring new microorganisms and their strains that adapt to and ferment beer wortis an important key factor in the rational development of new blends for brewers to ensure that the production of various beers and their flavors become reliable and reproducible. Based on these data, we conclude that this is a vast field that needs to be further explored,with potential for industrial applications and in the development of basic and applied science. Future work connecting the bacterial compositions of mixed cultures used for fermentation to metadata on the base wort, kinetics of fermentation, and organoleptic compounds produced in various sour beers will enable brewers to construct bespoke mixed cultures the generate desired sensorial results.

Supplementary Materials: The following are available online at www.mdpi.com/article/10.3390/fermentation7030174/s1, Supplementary Information S1: Raw reads, ASVs sequences, microorganisms' identification, and prevalence in each sample (Microsoft Excel file).

Author Contributions: Conceptualization, R.E.A.P. and M.L.B.; methodology, R.E.A.P. and M.L.B.; software, R.E.A.P., F.P.L.L. and M.L.B.; validation, R.E.A.P., F.P.L.L. and M.L.B.; formal analysis, R.E.A.P., F.P.L.L. and M.L.B.; investigation, R.E.A.P. and M.L.B.; resources, R.E.A.P., F.P.L.L. and M.L.B.; data curation, R.E.A.P. and M.L.B.; writing—original draft preparation, R.E.A.P.; writing—review and editing, R.E.A.P., F.P.L.L. and M.L.B.; visualization, R.E.A.P.; supervision, F.P.L.L. and M.L.B.; project administration, F.P.L.L. and M.L.B.; funding acquisition, R.E.A.P., F.P.L.L. and M.L.B. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data presented in this study are available in the Supplementary Information.

Conflicts of Interest: The authors declare no conflicts of interest.

Appendix A

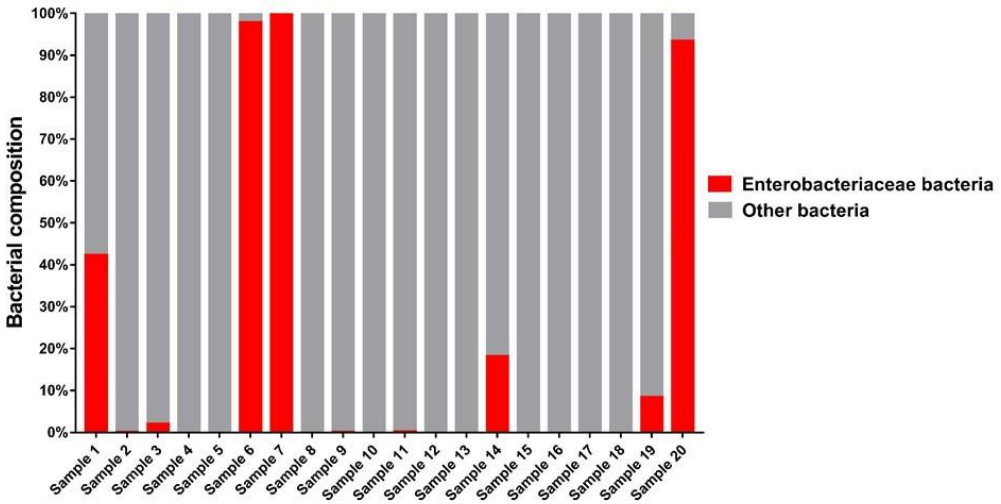

Figure A1. Enterobacteriaceae family presence in the bacterial composition of the analyzed samples.Bacteria from the Enterobacteriaceae family are commonly detected in the first fermentation phase of mixed-fermentation beers produced by spontaneous fermentation (De Roos and De Vuyst, 2019) and in our study they could be identified in relevant proportions in samples 1, 6, 7, 14, 19, and 20 (>8% of the ASVs detected in each sample).

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4.2. Manuscrito 2 - Isolation of wild yeasts from Olympic National Park and *Moniliella megachiliensis* ONP131 physiological characterization for beer fermentation

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Isolation of wild yeasts from Olympic National Park and *Moniliella megachiliensis* ONP131 physiological characterization for beer fermentation

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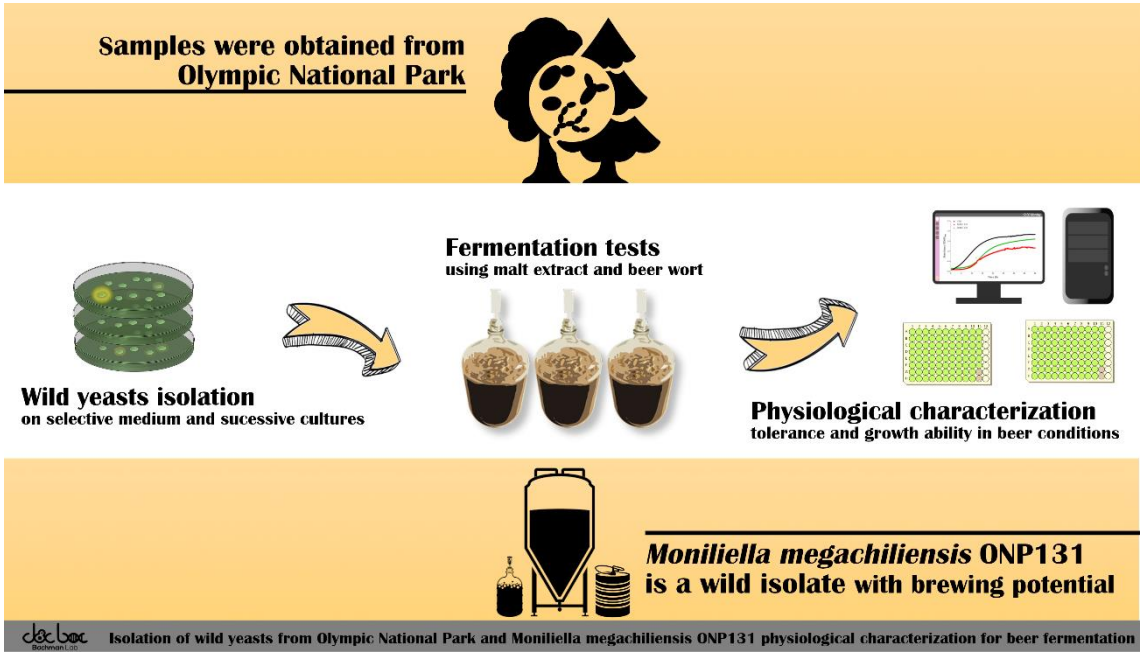
Graphical abstract



Abstract

Thousands of yeasts have the potential for industrial application, though many were initially considered contaminants in the beer industry. However, these organisms are currently considered important components in beers because they contribute with new flavors. Non-*Saccharomyces* wild yeasts can be important tools in the development of new products, and the objective of this work was to obtain and characterize novel yeast isolates for their ability to produce beer. Wild yeasts were isolated from environmental samples from Olympic National Park and analyzed for their ability to ferment malt extract medium and beer wort. Six different strains were isolated, of which *Moniliella megachiliensis* ONP131 displayed the highest levels of attenuation during fermentations. We found that *M. megachiliensis* could be propagated in common yeast media, tolerate incubation temperatures of 37°C and a pH of 2.5, and was able to grow in media containing maltose as the sole carbon source. Yeast cultivation was considerably impacted (p<0.05) by lactic acid, ethanol, and high concentrations of maltose, but ONP131 was tolerant to high

salinity and hop acid concentrations. This is one of the first physiological characterizations of *M. megachiliensis*, which has potential for the production of beer and other fermented beverages.

Keywords: wild yeast, *Moniliella megachiliensis*, physiological characterization, beer, brewing

Declarations

Funding

Not applicable

Conflicts of interest

The authors declare that there is no conflict of interest.

1. Introduction

Beer is produced using four main ingredients: malted grains (usually barley), water, hops, and yeast (mostly *Saccharomyces pastorianus* and *S. cerevisiae*) (Bokulich and Bamforth, 2013). Searching for new products, additional ingredients have been introduced to beer recipes, such as fruits, herbs, and wood. Additionally, brewers are exploring new combinations of traditional ingredients, for example the quantity and varieties of hops, malting different grains or the use of non-conventional/wild yeasts during fermentation (Donadini and Porretta, 2017). It is estimated that thousands of these strains, if not species, of wild yeasts have potential for industrial application (Gutiérrez et al., 2018). Among them, some species in the *Brettanomyces*, *Candida*, and *Pichia* genera were initially considered only as contaminants of beer and wine (Campbell, 2003). However they are currently considered important components in high value-added beers, as they can produce sought-after flavors and aromas during fermentation (Michel et al., 2016).

Yeasts are ubiquitous in the environment and are often isolated from sugar-rich sources, such as fruit, berries, and plant exudates (Rao et al., 2008; Tikka et al., 2013), with soil and some insects being natural reservoirs of fermentative yeasts (Barry et al., 2018; Osburn et al., 2016). Multiple techniques for the isolation of wild yeasts are already well stablished. However, the process becomes complex when there is a need to characterize these isolates before their application in fermentation processes. Various tests must be performed to determine characteristics such as alcohols tolerance, ability to metabolize different types and concentrations of carbohydrates, and survival in adverse conditions (e.g., pH, temperature) (Tikka et al., 2013). In the screening process for yeasts with potential in brewing industry, it is of great importance to identify the aromatic compounds produced during and after fermentation (e.g., esters, fusel alcohols, phenols), their flocculation and attenuation profiles, and especially their growth characteristics (Osburn et al., 2016). As with *S. cerevisiae*, their domestication becomes important, selecting and maintaining wild species to obtain variants that are able to develop in a controlled way, even under suboptimal conditions compared to those observed in their natural environment (Gallone et al., 2016; Steensels et al., 2019).

Thus, the objective of this work was to isolate wild yeasts from their natural habitat, selecting them for their application potential for beer production and physiologically characterizing them, aiming at the future domestication and establishment of the cultures as commercial starters for the brewing market. Here, one such strain, *Moniliella megachiliensis* ONP131, showed great promise for beer production. Wild yeasts

of the *Moniliella* genus, which is still under studied regarding its taxonomy and ecology, are often found in tropical ecosystems, present in flowers (Thoa et al., 2015), unprocessed foods, and insects (Lachance et al., 2001; Rosa et al., 2009). Currently, the biotechnological importance of these yeasts lies in the commercial production of erythritol, a natural four-carbon sugar alcohol that is a non-cariogenic sweetener (Thoa et al., 2015). *Moniliella megachiliensis* has been reported for its large erythritol production capacity (Thoa et al., 2015), including patent-protected processes (Ghislain et al., 2002). It has further been described that this yeast is able to metabolize large concentrations of glucose and maltose (Singh and Kumar, 2019), which are the main carbohydrates found in beer worts. Although used extensively for erythritol production, there are few studies regarding its application in other fermentation processes. We found that *Moniliella megachiliensis* ONP131 could be propagated using traditional yeast growth media, tolerated various stressors associated with beer fermentation, and produced beer with a pleasant organoleptic profile.

2. Material and Methods

2.1. Wild yeast isolation and identification

Fifteen samples from sources such as soil, tree bark, roots, leaves, and flowers were collected in Olympic National Park (Port Angeles, Washington, US) in August, 2019. Samples were stored in sterile plastic zip type bags (Whirl-Pak®, Madison, WI, USA) and stored at 4°C until processing. A small piece of 2 cm² of each sample was aseptically extracted and added to 5 mL of YPM8E5 medium (10 g/L yeast extract, 20 g/L peptone, 80 g/L maltose, 5% ethanol (v/v), 50 µg/mL kanamycin, 50 µg/mL chloramphenicol), and then incubated with agitation for 72 h at 30°C. A volume of 10 µL of each sample was used for microbial isolation on Wallerstein Laboratory Nutrient agar (WLN) medium, and plates were incubated as described above. Colonies with yeast morphology were plated again on YPD agar medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L dextrose and 20 g/L agar), inoculated into 10 mL of YPD medium and then visualized by phase contrast microscope at 1000x intension to determine morphology and purity. Saturated cultures of pure isolates were mixed with 30% glycerol and stored at -80°C to establish a culture bank.

For species identification, one colony of each isolate was cultivated in liquid YPD medium, and then the cell pellet from 200 µL of culture was used for genomic DNA (gDNA) extraction. Subsequently, 0.5 µL of gDNA was used as the template for PCR with primers NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') (Osburn et al., 2018) to amplify the variable domain (D1/D2) of the 26S rRNA gene, yielding PCR products of ~600 bp. PCRs were performed in an Eppendorf Mastercycler pro S thermocycler following the protocol: initial denaturation at 98°C for 5 min; 30 cycles of denaturation at 98°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min; and ending with a final extension at 72°C for 10 min. PCR results were visualized using 1% agarose gel electrophoresis for 1 h at 130 V.

Amplified fragments were purified using a PCR Purification kit (ThermoScientific, Walthan, MA), quantified using a Nanodrop spectrophotometer (ThermoScientific, Walthan, MA), and submitted for Sanger sequencing by ACGT Inc (Wheeling, IL). The sequencing results were analyzed and compared for sequence homology using the National Center for Biotechnology Information (NCBI) database and BLAST nucleotide tool, available at https://blast.ncbi.nlm.nih.gov.

2.2. Phylogenetic analysis

Sequencing of each amplified D1/D2 domain of the 26S rRNA gene was used to analyze the phylogenetic relationships among wild yeasts, using MEGA software (Molecular Evolutionary Genetics Analysis) v.10.1.7 (https://www.megasoftware.net) for alignment, construction and visualization of the phylogenetic tree (Tamura et al., 2021). Sequence alignments were performed using ClustalW, which was also used to generate the phylogenetic trees using the bootstrap method with 1000 replications through neighbor-joining statistical method, as done by Haile and Kang (2019). *Saccharomyces cerevisiae* WLP001 (White Labs, San Diego, CA) was used as a reference yeast, since it was a worldwide commercially available strain, repeatedly applied in molecular analysis for yeasts comparison and fermentation trials.

2.3. Fermentation tests

Isolated yeasts were inoculated into two tubes containing 5 mL YPD medium and incubated under agitation for 48 h at 30°C until obtaining a density of approximately 10^9 CFU/mL. Viable cells (CFU/mL) were counted by serial dilutions using saline solution (0,9% NaCl) and plating in agar YPD medium, following a 48 h incubation period at 30 °C. One of the cultures was used to inoculate 400 mL of 100% Pilsner malt extract (Briess) medium, density 1.040 g/cm³ and pH 5.4, while the second was used to inoculate 400 mL of India Pale Ale beer wort, 1.049 g/cm³, pH 5.5, 65 IBU, produced and supplied by Upland Brewering Company (Bloomington, IN, USA). Fermentation flasks were incubated without agitation, with an air-lock to release CO_2, for 14 days at room temperature (approximately 23°C). During fermentation, whether visible fermentation activity (e.g., gas release through the air-lock) and the formation of a biofilm on the liquid surface was observed. After 14 days, samples were collected to analyze the final density and pH, allowing observation of acidification ability and apparent attenuation by each wild yeast isolated. Density was analyzed using a MISCO digital refractometer and pH using an Accumet AB150 (Thermo Fischer Scientific) pH meter. Uninoculated malt extract and beer wort solutions were kept throughout the test to observe any possible undesirable fermentation by microorganisms that did not originate exclusively from the collected samples.

2.4. Evaluation of important brewing characteristics of *M. megachiliensis* isolate ONP131

M. megachiliensis ONP131 and the commercial ale yeast *S. cerevisiae* WLP001 (White Labs, San Diego, CA) were inoculated in 5 mL of YPD medium (pH 5.4), and incubated overnight with agitation at 30 °C. The optical density of both cultures was evaluated using a Beckman Coulter DU730 UV/Vis Spectrophotometer, and then cultures were diluted with ultrapure water to an OD_{660nm} of 0.06. Tests for the characterization of *M. megachiliensis* ONP131 were performed in 96-well plates, in duplicate, three independent times, as done previously by Rogers et al. (2016). For each well, 100 µL of the dilution of each yeast was mixed with 100 µL of the medium to be tested, at a concentration of 2x, and overlaid with 50 µL of sterile mineral oil to avoid evaporation. Growth curves were followed over 48 h at 30°C by optical density readings every 15 min using a Synergy H1 Plate Reader (Biotek, Winooskim VT) with Gen5

Microplate Reader and Imager Software (Biotek, Winooski, VT). For all tests *S. cerevisiae* WLP001 was used as control yeast, and uninoculated media were used as blank (negative control).

To evaluate important characteristics for use in beer production, different treatments were tested. Initially, the growth of *M. megachiliensis* was evaluated in standard YPD medium, as well as in dry malt extract (DME), with densities of 1.020 g/cm³ and 1.040 g/cm³, respectively. Cultures in YPD were subjected to different incubation temperatures at 30°C, 34°C, and 37°C, seeking to assess tolerance to higher temperatures for growth. Consumption of different carbohydrates was tested in the presence of 2% (w/v) glucose, 2% maltose, 2% sucrose, or 2% galactose, while tolerance to osmotic stress was assessed using concentrations of 10, 20 and 30% (w/v) glucose and maltose. Carbohydrates consumption and osmotic stress experiments were performed with YP as a basis for medium formulation (10 g/L yeast extract and 20 g/L peptone). Tolerance to salinity was tested using YPD + 0.5, 1, 5, or 10% (w/v) NaCl. Ethanol tolerance was evaluated in YPD medium supplemented with 2, 4, 5, or 6% (v/v) ethanol. As a control, tests were performed using only YPD to evaluate the growth kinetics and final OD_{660nm} of *M. megachiliensis* and *S. cerevisiae* cultures.

The ability to grow at different pHs was evaluated by incubating *M. megachiliensis* in YPD medium at pH = 2.5, 3.0, 3.5, 5.0 (adjusted with 1 M HCl) or pH = 8.0 (adjusted with 1 M NaOH). To observe the impact of lactic acid on yeast cultivation, YPD medium was adjusted with 85% lactic acid (to a final pH of 2.5, 3.0, 3.5, or 5.0 prior to yeast inoculation). The ability to tolerate different concentrations of iso-α-acids (compounds present in hops that have antimicrobial activity) was also evaluated in YPD medium, with concentrations ranging from 10 to 200 ppm of 30% isomerized hop extract (Hopsteiner, Mainburg, DE). Spot tests were performed in duplicates to confirm tolerance to hop compounds, following an adaptation of the protocol done in Giannakou et al. (2021). Briefly, *M. megachiliensis* and *S. cerevisiae* were cultured overnight in YPD medium under agitation, then 10-fold serial dilutions were made from these cultures using saline solution (0,9% NaCl), starting at 10^8 CFU/mL and finishing at 10^3 CFU/mL. For the test, 5 µL of each culture dilution was applied in YPD agar plates supplemented with (a) 30% isomerized hop extract at concentrations of 90 and 120 IBU (International Bitterness Units) or (b) β-acids, from 45% Beta Bio (Hopsteiner, Mainburg, DE) at concentrations of 100 ppm and 200 ppm, which were subsequently incubated over 48 h at 30 °C.

2.5. Statistical analyses

All statistical analyses were performed using GraphPad Prism 7 software. Differences between values were compared using analysis of variance (ANOVA) and Tukey test, where *p*-values < 0.05 were considered statistically significant.

3. Results

3.1. Wild yeasts from Olympic National Park samples

Sixteen yeast isolates were obtained from fifteen samples collected in Olympic National Park. All isolates were subjected to fermentation tests in malt extract and beer wort. Yeasts displaying the most promising fermentative potential were identified by PCR using NL1/NL4 primers, a primer set routinely

used by several researchers for wild yeasts identification after their isolation from natural sources (Barry et al., 2018; Thanh and Hien, 2019; Zhao et al., 2021). Six strains from four different species were identified (more information about similarities with GenBank database sequences are shown in Table S1): *Debaryomyces hansenii* (ONP21, GenBank accession n. MZ506604; ONP27, GenBank accession n. MZ506605), *Yamadazyma scolity* (ONP63, GenBank accession n. MZ506606), *M. megachiliensis* (ONP93, GenBank accession n. MZ506607; ONP131, GenBank accession n. MZ506609), *Starmerella riodocensis* (ONP96, GenBank accession n. MZ506608). We observed that the colony morphologies of the *D. hanseniii*, *S. riodocensis* and *Y. scolity* isolates on WLN and YPD plates were similar to *S. cerevisiae* WLP001, being circular with smooth margins, though they did not show elevation (Supplementary Information – Fig. S1). In contrast, *M. megachiliensis* ONP93 and ONP131 formed colonies with filamentous borders, easily distinguishable from the other yeasts for having an olivaceous black color after 72 h of growth. Microscopic analysis showed cells with almost twice the size of *S. cerevisiae*, that formed clumps. After 24 h of aerated cultivation in liquid YPD medium, we also observed that *M. megachiliensis* isolates sedimented as popcorn-like clumps after 10 s without agitation (Fig. S2).

Based on the D1/D2 sequences of the 26S rRNA genes of the above yeasts, a phylogenetic tree was constructed to determine relationships among the wild isolates and the *S. cerevisiae* WLP001 yeast (GenBank accession n. MZ506610) (Fig. 1). The yeasts *D. hansenii* and *Y. scolity* are taxonomically classified in the same family, Debaryomycetaceae (which explains their proximity in phylogeny), and compared to *S. cerevisiae* and *S. riodocensis*, they all belong to same class of Saccharomycetes. This class comprises those considered "true yeasts", which share ultrastructural characteristics such as aspects of nuclear division and ascospore formation (Blackwell and Spatafora, 2004). All of the above mentioned yeasts are also part of the Ascomycota phylum, while *M. megachiliensis* is classified in the phylum Basidiomycota, which explains its phylogenetic distance from the others.

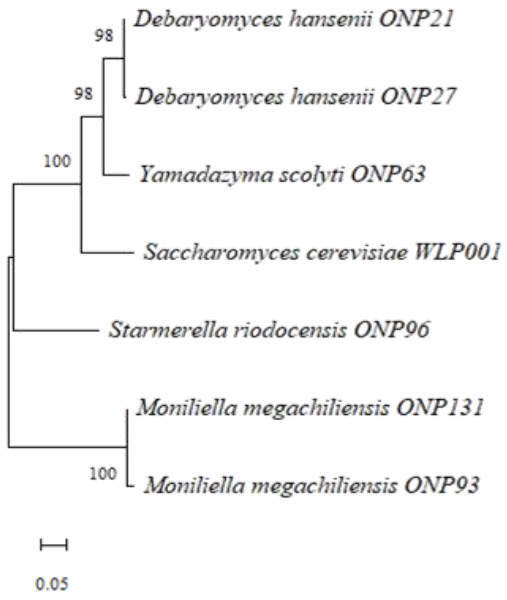


Fig. 1: Phylogenetic tree of wild yeasts identified from Olympic National Park samples and a commercially available *S. cerevisiae* yeast. The D1/D2 rRNA sequences of the isolated strains were aligned, and the phylogenetic relationships among them were constructed using the bootstrap method with 1000 replicates, based on neighbor-joining analysis.

3.2. Fermentation tests with the wild yeasts isolates

Fermentation tests results are shown in Table 1. In malt extract and beer wort fermentations by *D. hansenii* ONP21 and ONP27, we observed that, in addition to the final pH and density being higher than ideal for beers, production of non-pleasant aromas that are undesirable for fermented beverages were detected. Although off-flavors were not noticed in *Y. scolity* ONP63 and *S. riodocensis* ONP96 fermentations, their inability to adapt to beer wort suggests that these isolates are not suitable for application in this fermentation process. *M. megachiliensis* isolates ONP93 and ONP131 fermentations displayed final pH, density, and alcohol by volume (ABV) values closer to those found in fermentations by *S. cerevisiae* WLP001, in addition to presenting interesting flavors similar to those detected in beers with esterified aromas.

Table 1: Fermentation tests of malt extract and beer wort using wild yeasts isolated from Olympic National Park. Preliminary sensory notes were collected as described previously (Osburn, Ahmad, and Bochman 2016).

Strains	Malt extract fermentation[a]					Beer wort fermentation[b]					Sensory notes
	Final pH	Final gravity	ABV	Biofilm[d]	Activity in fermentation[c]	Final pH	Final gravity	ABV	Biofilm[d]	Activity in fermentation[c]	
D. hansenii ONP21	5.08	1.034	0.8%	NO	NO	4.59	1.044	0.69%	NO	YES	Sulfur, butyric, mercaptan
D. hansenii ONP27	5.14	1.039	0.14%	NO	NO	4.47	1.046	0.41%	NO	YES	Sulfur, butyric, mercaptan
Y. scolyti ONP63	4.48	1.038	0.27%	NO	NO	5.08	1.047	0.28%	NO	NO	Sweet, honey-like
M. megachiliensis ONP93	4.26	1.023	2.27%	YES	YES	4.12	1.029	2.72%	YES	YES	Ester, phenolic, bubble gum
S. riodocensis ONP96	4.30	1.036	0.54%	NO	NO	4.84	1.046	0.41%	NO	NO	Sweet, honey-like
M. megachiliensis ONP131	4.06	1.020	2.66%	YES	YES	4.30	1.034	2.05%	YES	YES	Ester, phenolic, bubble gum
S. cerevisiae WLP001	4.30	1.013	3.57%	NO	YES	4.28	1.012	4.94%	NO	YES	Neutral, slightly fruity

[a] The original gravity of the malt extract solution was 1.040 and initial pH of 5.40.
[b] The original gravity of the beer wort was 1.049 and initial pH of 5.50.
[c] Activity in fermentation refers to gas production observed through the airlock, bubble formation visible in the liquid culture, or foam at the liquid surface.
[d] Biofilm or "pellicle" is the aggregation of cells through proteins and polysaccharide bonds at the liquid surface.
Abbreviations: ABV –alcohol by volume

In Figure 2, we show that *M. megachiliensis* isolates displayed an apparent attenuation of carbohydrates present in malt extract and beer wort between 30 and 50%, demonstrating a 3 to 4-fold greater capacity to attenuate wort compared to the other isolates. Because the *M. megachiliensis* ONP131 isolate

performed slightly better than ONP93 and was informally ranked as producing better beer, it was selected for subsequent tests and physiological characterization.

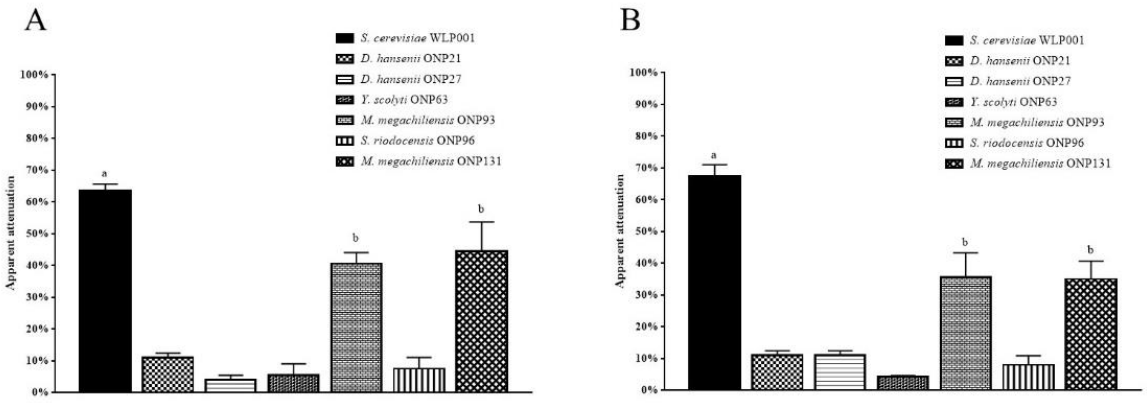


Fig. 2: Apparent attenuation in malt extract (A) and beer wort (B) by the isolated yeasts. Commercial available *S. cerevisiae* was used as a control for normal attenuation by brewer's yeast. Error bars indicate ± SD. Letters indicate statistical difference ($p < 0.05$): **a**) difference from all yeasts; **b**) different from all yeasts, except for the same yeast species.

3.3. Characterization of *M. megachiliensis* ONP131 brewing characteristics

The growth capacity of *M. megachiliensis* isolate ONP131 was evaluated under different conditions and compared to a standard ale strain of *S. cerevisiae.* Growth curves are presented in Figure S3, while the following results are related to the final OD_{660nm} measured in *M. megachiliensis* ONP131 and *S. cerevisiae* WLP001 cultures.

No significant differences were observed in the absorbance of the two yeasts when cultivated in YPD medium ($p < 0.05$) (Fig. 3A), but there was a tendency of a longer lag phase when *M. megachiliensis* was cultivated in DME with densities of 1.020 and 1.040 (Fig. S3), showing lower final absorbances in both cases. The incubation temperature directly influenced the capacity of *M. megachiliensis* to flourish, demonstrating a decrease of approximately 40% in final absorbance of cultures at 34°C and 37 °C relative to 30°C (Fig. 3B).

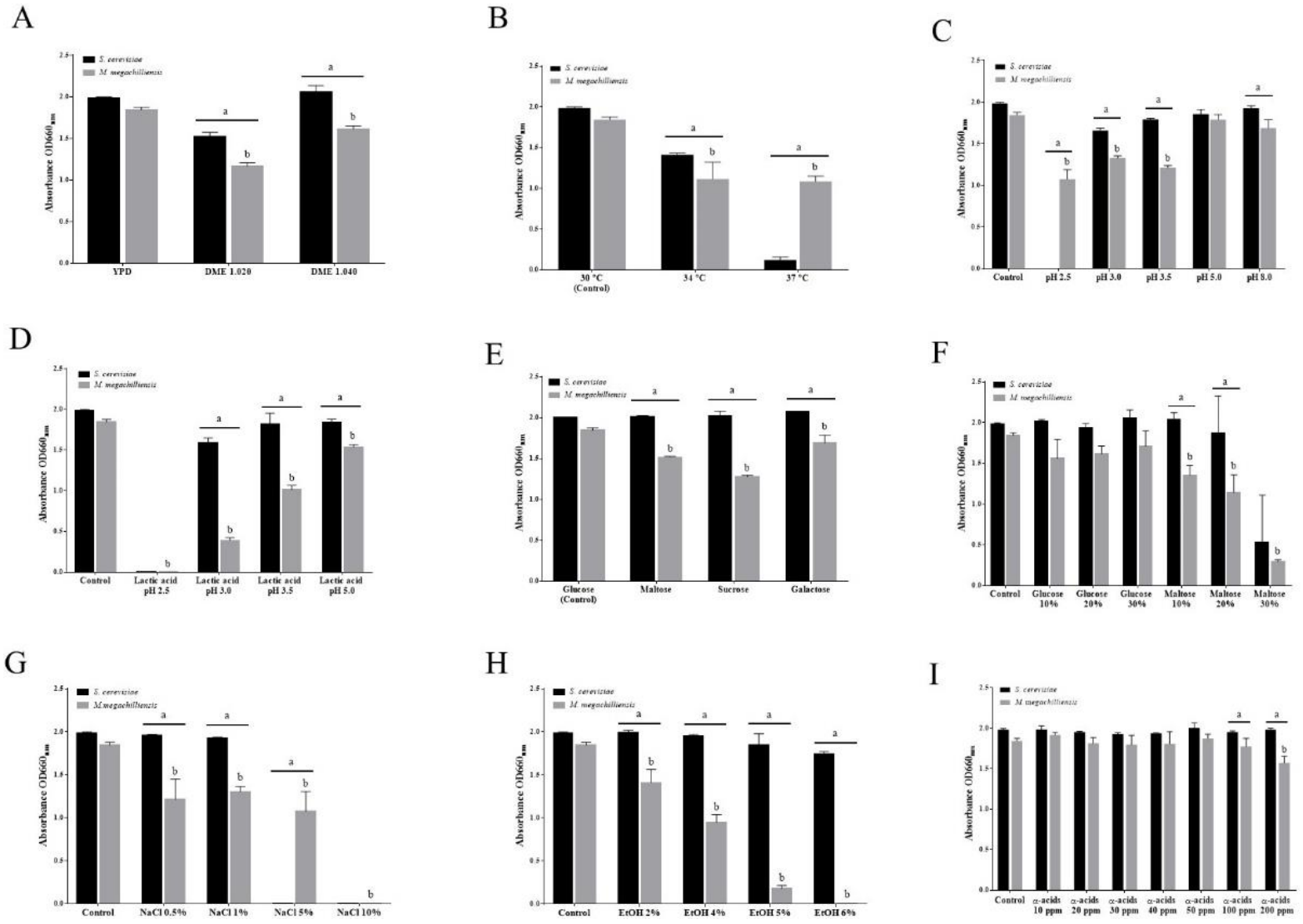


Fig. 3: *M. megachiliensis* physiological characterization for beer fermentation. Characteristics of *M. megachiliensis* ONP131 regarding growth medium for yeast propagation (A), incubation temperature (B), initial culture pH (C), tolerance to lactic acid (D), growth ability in media containing other carbohydrates as a carbon source (E), osmotic tolerance to glucose and maltose (10% > 30%) (F), halotolerance in NaCl-supplemented media (G), ethanol tolerance (H), and resistance to α-acids from hops extract (I). Data are expressed as the terminal $O.D._{660nm}$ of the cultures. Error bars indicate ± SD. Letters indicate statistical differences ($p < 0.05$): **a**) difference between the growth of *S. cerevisiae* WLP001 (control yeast) and *M. megachiliensis* cultures; **b**) differences between the control group and analyzed value. **Control:** yeast growth analyzed in YPD medium pH 5.5, with no NaCl, α-acids, lactic acid, HCl, or ethanol added, with a 30 °C incubation temperature.

Medium acidification with HCl to pH 2.5 hindered the growth of both *S. cerevisiae* and *M. megachiliensis*, but ONP131 maintained an $OD_{660nm} \geq 1.0$, representing at least 50% of its original growth observed in control culture (pH 6.0) (Fig. 3C). Although a significant ($p < 0.05$) decrease in growth was observed in the range of pH 2.5-3.5, cultures subjected to pH 5.0 and 8.0 displayed growth equivalent to

that of the control. Acidifying the culture medium with lactic acid proved to be more harmful to yeast growth, preventing it at pH 2.5 or limiting it to an absorbance ≤ 1.0. Even the medium with low amounts of added lactic acid (pH 5.0) was also harmful to the growth of both yeasts (Fig. 3D).

While comparing the growth of *M. megachiliensis* and S. cerevisiae, we next assessed the effects of various sugars. In general, other carbohydrate sources are not metabolized by yeast as effectively as glucose. When applied at a concentration of 2%, maltose, sucrose and galactose resulted in cultures with a lower absorbance (OD_{660nm} = 1.51, 1.27, and 1.68, respectively) than that detected for the control medium containing glucose (OD_{660nm} = 1.84) and for *S. cerevisiae* (Fig. 3E). Osmotic pressure tolerance analysis using glucose and maltose revealed that *M. megachiliensis* tolerates glucose concentrations up to 30%, having no significant differences in final optical density ($p < 0.05$) (Fig. 3F). However, concentrations above 10% maltose caused changes in growth, with 30% maltose significantly limiting growth to OD_{660nm} = 0.3, which is only 15% of that observed in the control YPD medium.

Halotolerance is a characteristic of some yeasts, and this ability to tolerate high salt concentrations was also evaluated in our study. We found that *M. megachiliensis* tolerated up to 5% NaCl, while the same concentration greatly limited the growth of *S. cerevisiae* (Fig. 3G). Supplementation of the culture medium with ethanol next revealed that ONP131 growth decreased as ethanol concentration increased up to 6% ethanol, which completely prevented *M. megachiliensis* growth. (Fig. 3H). Among limiting conditions for growth in beer wort, the α-acids derived from hops are known to inhibit bacterial growth but can also affect yeasts at high concentrations (Hazelwood, 2010). In our study we observed that only very high concentrations (≥ 200 ppm) of α-acids were able to impact *M. megachiliensis* growth (Fig. 3I); below that, no statistical differences were observed compared to control cultures. Tolerance was also observed for α-acids (90 and 120 IBU) and β-acids (100 ppm and 200 ppm) used as supplements in solid culture media, in which *M. megachiliensis* showed similar growth to *S. cerevisiae*, demonstrating resistance to these conditions (Fig. 4) and suggesting *M. megachiliensis* tolerance to the antimicrobial components present in hops.

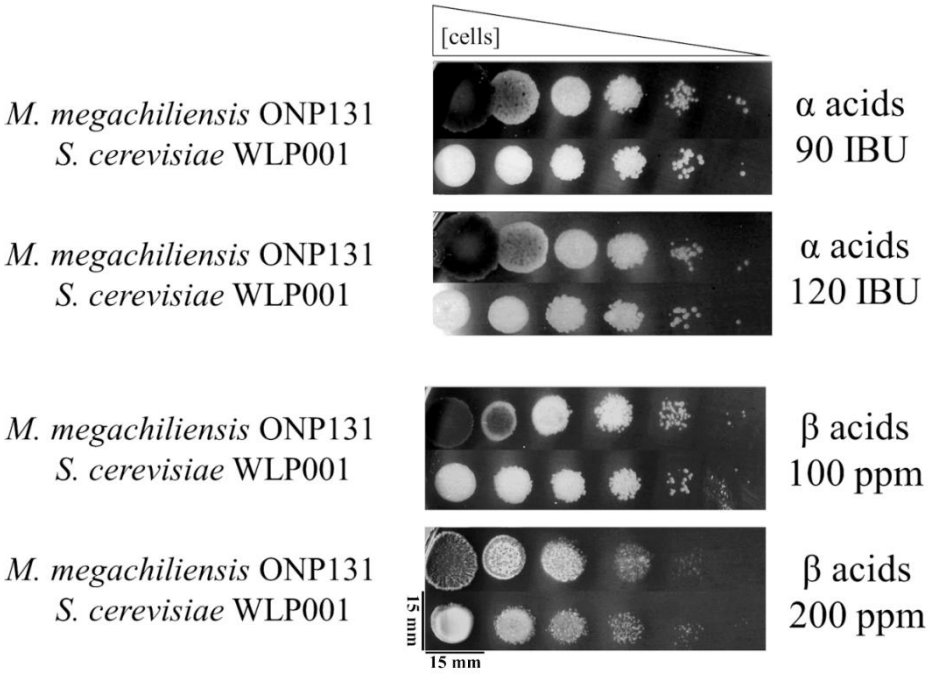


Fig. 4: *M. megachiliensis* tolerance to α- and β-acids from hops. *M. megachiliensis* ONP131 and *S. cerevisiae* WLP001 (control) were cultured on YPD plates supplemented with high concentrations (90 or 120 IBU) of iso-α-acids (30% isomerized hop extract) or (100 ppm and 200 ppm) β-acids (45% Beta Bio). Plates were photographed after an incubation period of 48 h at 30 °C. Images are presented at the same resolution and magnification. The experiment was repeated twice, with similar outcomes.

4. Discussion

When bio-prospecting, yeasts are generally found associated with bees and flowers (Lachance et al., 2001; Pimentel et al., 2005) as well as from a wide range of other sources, such as processed food products, soil, marine environments, and fermented products (Suzuki et al., 2011). Here, we isolated novel strains from small plant roots, tree bark and flowers, which are sources similar to those described elsewhere in the literature. Of the six strains displaying some fermentative potential, low metabolism of wort sugars was observed for the ONP21 and ONP27 isolates of *D. hansenii*, yielding apparent attenuation between 5 and 10% in both tests. Used in many processes such as the fermentation of cacao and meat products (Ramos et al., 2017), *D. hansenii* is known to have great biotechnological potential. Concerning its application in the brewing industry, some strains have interesting features, such as conversion of ferulic acid to 4-vinyl-guaiacol (Yaguchi et al., 2017). However, production of unpleasant aromas by ONP21 and ONP27 such as sulfur, butyric acid, and mercaptans was easily detectable by simple sensory analysis in our study, which was considered a problem for their future application in beer fermentation.

The inability of *S. riodocensis* ONP96 to utilize maltose for its development (Pimentel et al., 2005) was detected in both fermentation tests, resulting in an apparent attenuation of 5-10%. *Y. scolity* has been reported to be able to grow using maltose, however the apparent attenuation observed suggested it was unable to ferment this carbohydrate in our study (apparent attenuation between 3 and 10%) and others (Kurtzman, 2011). Our results also indicated that neither *Y. scolity* ONP63 nor *S. riodocensis* ONP96 contributed to the aroma *bouquet* of fermented malt extract or beer wort, suggesting that these strains have no potential for application in the fermentation of beers even when co-inoculated with strains better able to metabolize maltose. This left the *M. megachiliensis* isolates to test further.

Despite the fact that the relationship between the classes of fungi within the Basidiomycota phylum is subject of discussion, the *Moniliella* genus represents yeast-like fungi that can be identified by ellipsoidal budding cells that form terminally on true hyphae, pseudomycelium, or chlamydospores and which are capable of fermenting various sugars, including maltose (Blackwell and Spatafora, 2004; Rosa et al., 2009; Singh and Kumar, 2019). As for this writing, there are no reports in the literature related to the application of *M. megachiliensis* in fermented beverage production, so it was necessary to characterize its fermentation capacity, as well as the flavors and aromas originating from fermentation. Although they do not have an attenuation profile like *S. cerevisiae,* it is known that yeasts with a low or medium attenuation percentage can have a strong contribution to the flavors of a beer (Michel et al., 2016). The esterified and bubble gum aromas observed in our study are in agreement with the work by Hoog et al. (2011) and Thanh and Hien, (2019), in which they report "fruity" and "peach-like" aromas derived from ethyl-acetate, γ-decalactone, and acetaldehyde in media cultivated with yeasts of the *Moniliella* genus. Thus, because it was responsible for the production of pleasant aromas and had moderate attenuation capacity, our *M. megachiliensis* ONP131 isolate was selected for further study. Even with a black-pigmented colony, in preliminary tests in which *M. megachiliensis* was used to ferment worts with low Lovibond units (2-6 units = yellow/golden beers), beer color remained unchanged after 1 month of bottling and similar to the control (beer produced with *S. cerevisiae*).

The biofilm presented on the liquid surface of *M. megachiliensis* fermentation flask was formed by a thin layer of cells, which easily decanted after 2 days of refrigeration at 4 °C. Although biofilm formation is a concern for some breweries, other biofilm producing microorganisms (e.g. *Brettanomyces* yeasts and lactic acid bacteria) are widely used by many brewers around the world, mainly in beer aging processes (Piraine et al., 2021; Riedl et al., 2019). Thus, *M. megachiliensis* can be applied using the same control methods used in these processes. Due to limited resistance to low concentrations of ethanol (~7%) and acetic acid (0.5%) already demonstrated in some strains of *M. megachiliensis*, it is suggested that cross-contaminations can easily be controlled in industrial areas where this yeast is unwanted, using traditional sanitization procedures (Nakayama et al., 2016)**.**

There is a need to know the performance of wild yeasts through their domestication to establish the feasibility of using these organisms for brewing purposes (Steensels and Verstrepen, 2014). As described by Rakete and Glomb (2013), 90% of beer wort is composed of glucose, sucrose, maltose, and maltotriose, usually with a higher concentration of maltose. Therefore, tests were performed to characterize yeast growth using mainly the carbohydrates present in wort, in which we observed the ability of *M.*

megachiliensis to grow in media where these carbohydrates were used as the sole carbon source. The possibility of cultivating yeast in DME also allows homebrewers and craft breweries to propagate this isolate in-house and maintain successive controlled fermentations.

Thermotolerance in yeasts is an uncommon feature, but those able to withstand temperatures ≥ 37°C are of interest for industrial use because they require lower costs for bioprocesses cooling and reduce contamination risks in these systems (Lehnen et al., 2019). We found that *M. megachiliensis* was able to sustain its growth when incubated in the 34-37°C range, even though the final absorbance value was lower than that observed for growth at 30°C. Therefore, this yeast has potential for application in bioprocesses that demand higher temperatures, as has already been demonstrated for the thermotolerant yeasts *Kluyveromyces marxianus* and *Ogataea polymorpha* (Lehnen et al., 2019).

Increases in osmotic pressure cause changes in the viability, size, and shape of lager and ale yeasts, which influence their fermentation capacity (Pratt et al., 2003). Osmotolerance is strain-dependent, being derived from membrane structure, vacuolar function, residual levels of trehalose, and especially the abundance of osmoprotective macromolecules (Gibson et al., 2007). In media containing 10, 20 or 30% glucose, no statistical difference was observed in the final absorbance of the *M. megachiliensis* cultures. However, when the same maltose concentrations were analyzed, it was observed that there was a decrease in OD_{660nm} due to high osmotic pressure and inability to metabolize this carbohydrate. Although different enzymatic pathways are activated in response to high extracellular osmolarity (e.g., the high-osmolarity glycerol pathway), at certain concentrations, these are not sufficient to ensure cell development, consequently culminating in a decrease of cell viability and concentration (Gibson et al., 2007).

Exploring the ability of yeasts to tolerate salt concentrations (ionic stress) is important in industrial fermentations, where salt can favor yeast growth, enhance ethanol production, and at the same time, reduce the risk of contamination by microorganisms with low halotolerance (Corte et al., 2006). While *S. cerevisiae* was not able to grow in 5% NaCl, *M. megachiliensis* sustained its growth and displayed halotolerance up to 5% NaCl with no significant difference in absorbance compared to growth with at 0.5% and 1% NaCl. At 10% NaCl concentration, however, the yeast was not able to survive.

Ethanol inhibits cell growth, viability, and fermentation rate in yeast (Pratt et al., 2003). Yeast ethanol tolerance can be assessed by exposing the yeast culture to ethanol at different concentrations until cellular growth suppression occurs, an important tool to characterize yeast species and strains considered for application in alcoholic fermentations (Da Silva et al., 2013). We observed that the growth of *M. megachiliensis* decreased with increasing ethanol concentration in the medium, which is similar to the pattern displayed by other wild yeast isolates characterized by Lee et al. (2011). Ethanol at a 6% concentration was identified as a limiting factor for *M. megachiliensis* ONP131 growth, which has similarly been described as a limiting factor for several other wild yeasts, such as *Hanseniaspora uvarum*, *Lachancea kluyveri*, and *Torulaspora delbrueckii* (Osburn et al., 2016). Therefore, it is suggested that *M. megachiliensis* is unable to survive in fermentations that exceed this ethanol concentration and should be used to produce beer below 5% ethanol, though adaptation to increasing ethanol concentration during fermentation (rather than immediate exposure to 6% ethanol upon inoculation) may enable *M. megachiliensis* ONP131 to survive in higher ABV beers.

It is well known that pH is another limiting factor for the proper growth of microorganisms, as well as the kinetics of pH decrease during fermentations by some microorganisms (Narayanan et al., 2016). Even though yeast cells are able to maintain appropriate internal pH by utilizing cell buffer systems and other pathways during changes in external pH conditions, certain pH ranges are not tolerated by yeasts (Brandão et al., 2014). In our tests, we found that *M. megachiliensis* was able to maintain its growth in YPD medium adjusted to pH 2.5 with HCl, but when the pH was adjusted to 2.5 with lactic acid, growth was not observed. The acidic shock occasioned by high levels of lactic acid was also observed by Rogers et al. (2016) for *S. cerevisiae*, which generally fail to carbonate sour beers with low pHs. The ability to withstand a pH range ≥ 3.0 (in YPD adjusted with HCl or lactic acid) suggests that application of *M. megachiliensis* ONP131 in sour beers (pH ~3.4) is an interesting option, whether for complete wort fermentation or post-fermentation, during the natural carbonation process.

Due to large concentration of hops in certain beer styles (e.g., India Pale Ales), we were also interested in determining the capacity of *M. megachiliensis* to tolerate compounds present in hops, such as α- and β- acids. As demonstrated by Methner et al. (2019) and Michel et al. (2016), the resulting inhibitory effect of these compounds may vary according to the yeast species analyzed, of which not all wild isolates are able to grow in concentrations up to 100 IBU and 200 ppm of β-acids. Here, though, we found that *M. megachiliensis* was able to maintain its growth at levels close to that observed for the control (no α-acids added), as well as able to tolerate β-acids concentrations between 100-200 ppm. Thus, we identified hop tolerance by the ONP131 isolate as evidence that it can be used in the production of highly hopped beers.

While only a short list of yeasts is currently considered GRAS (*Generally Regarded as Safe)* or QPS (*Qualified Presumption of Safety*) for use in food production, several tests are needed to confirm new microorganisms as safe starter cultures in these bioprocesses (Capece et al., 2018; Gibson et al., 2017; Ricci et al., 2017). *Moniliella megachilliensis* is not a member of these lists, however there are no reports in the literature about diseases or infections caused by this yeast. Nevertheless, more studies regarding the safety of its application must be implemented, since strains classified in *Moniliella* genus (specifically *M. suaveolens*) have already been listed as possible opportunistic and responsible for subcutaneous infections in immunocompromised animals and humans (de Hoog et al., 2011). Tests to evaluate *M. megachiliensis* resistance to antimicrobial compounds will also be conducted prior to its industrial application, as this characteristic allows predicting the effectiveness in diseases prevention and treatment of fungal infections (Fernández-Pacheco et al., 2021).

Another relevant aspect regarding safety is the production of biogenic amines during enzymatic decarboxylation of amino acids present in the beer wort (Koller, 2020). Biogenic amines are nitrogen compounds with low molecular mass that, when ingested in excess, can cause headaches, hypertension, nausea, and other symptoms that reveal their toxic effects (Wang et al., 2021). Certain strains of yeasts and bacteria that are generally used in mixed-fermentation beers are recognized as potent producers of biogenic amines when subjected to stress, thereby knowing the capacity of microorganisms to form these compounds is also important to ensure safety in their applicability (Caruso et al., 2002; Vejarano and Gil-Calderón, 2021). There are currently no studies on the formation of biogenic amines by yeasts of the *Moniliella* genus, thus special attention will be given in further studies to explore the safety of *M. megachiliensis* in this and other aspects.

5. Conclusion

In this work, we successfully isolated different yeast species from environmental samples collected in Olympic National Park. Upon analysis of fermentative activity by these yeast isolates, we selected *M. megachiliensis* ONP131 for further physiological characterization, of its growth in conditions generally found in beer worts. Overall, we found that *M. megachiliensis* ONP131 is an isolate with potential for the brewing industry, perhaps when co-inoculated with a typical *Saccharomyces* beer strain to overcome the low attenuation of ONP131. Based on its halo- and thermotolerance, *M. megachiliensis* ONP131 may also be of interest for other bioprocesses as well.

6. References

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Supplementary Information

Table S1: BlastN results used to identify the wild yeasts isolated from Olympic National Park samples.

Isolate	Top hit	NCBI accession n.	Identity (%)	Query cover (%)
ONP21	*Debaryomyces hansenii* strain YB01 large subunit ribosomal RNA gene, partial sequence	MH423839.1	99.30	98.00
ONP27	*Debaryomyces hansenii* large subunit ribosomal RNA gene, partial sequence	MT328058.1	99.65	96.00
ONP63	*Yamadazyma scolyti* voucher UCASIM-1236 28S ribosomal RNA gene, partial sequence	MH794404.1	99.60	90.00
ONP93	*Moniliella megachiliensis* strain 11-1074 26S ribosomal RNA gene, partial sequence	JX974360.1	96.65	94.00
ONP96	*Starmerella riodocensis* CBS 10087 28S rRNA gene, partial sequence; from TYPE material	NG_060377.1	97.66	95.00
ONP131	*Moniliella megachiliensis* strain CBS190.92 large subunit ribosomal RNA gene, partial sequence	EF137916.1	95.54	97.00

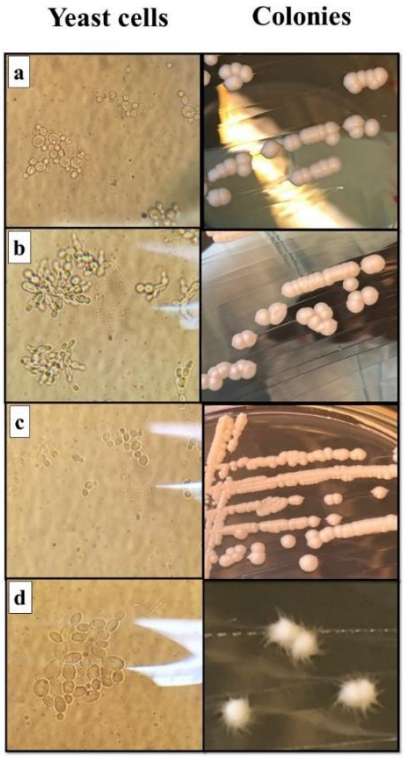

Fig. S1: Micrographs of isolated yeasts, taken under at x1000 magnification (left), and their respective colony morphologies (right), after 48 h on YPD agar at 30°C. a) *D. hansenii*, b) *Y. scolyti*, c) *S. riodocensis* and d) *M. megachiliensis*.

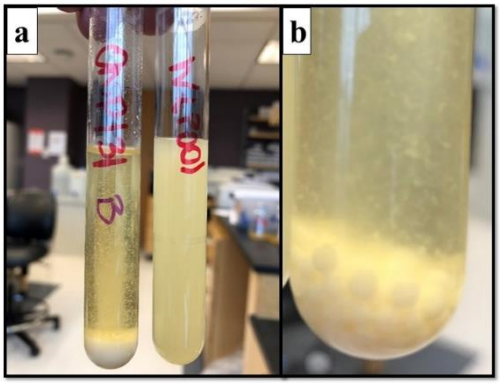

Fig. S2: Sedimentation of *M. megachiliensis* cultures after 10 s with no shaking. a) Yeast cultures after 24 h in 10 mL of YPD. The tube on the right is a *S. cerevisiae* WLP001 control culture. b) Popcorn-like clumps of sedimented ONP131 cells.

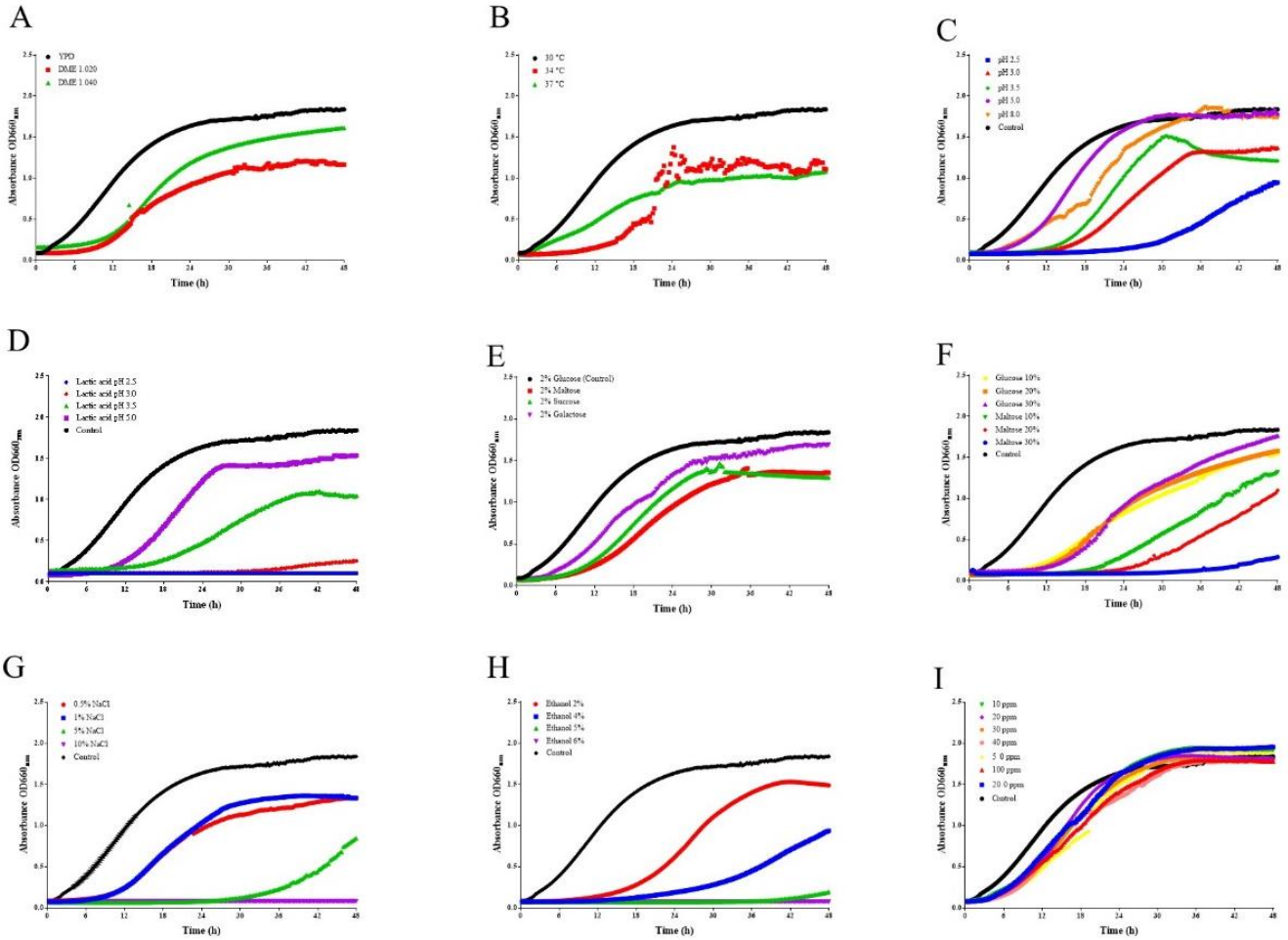

Fig. S3: Growth curves of *M. megachiliensis* ONP131 cultivated under different conditions. Medium for yeast propagation (A), incubation temperature (B), initial culture pH (C), tolerance to lactic acid (D), growth in media containing other carbohydrates as a carbon source (E), osmotic tolerance to glucose and maltose (10 to 30%) (F), halotolerance in NaCl-supplemented media (G), ethanol tolerance (H), and resistance to α-acids from hops extract (I). Growth curves were followed over 48 h at 30°C by optical density readings every 15 min on a Synergy H1 Plate Reader (Biotek, Winooskim VT) with Gen5 Microplate Reader and Imager Software (Biotek, Winooski, VT). Averaged data are expressed in absorbance (OD_{660nm}) values by time. Error bars representing standard deviations were omitted for data clarity.

4.3. Manuscrito 3 - Brewing and probiotic potential activity of wild yeasts *Hanseniaspora uvarum* PIT001, *Pichia kluyveri* LAR001 and *Candida intermedia* ORQ001

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Brewing and probiotic potential activity of wild yeasts *Hanseniaspora uvarum* PIT001, *Pichia kluyveri* LAR001 and *Candida intermedia* ORQ001

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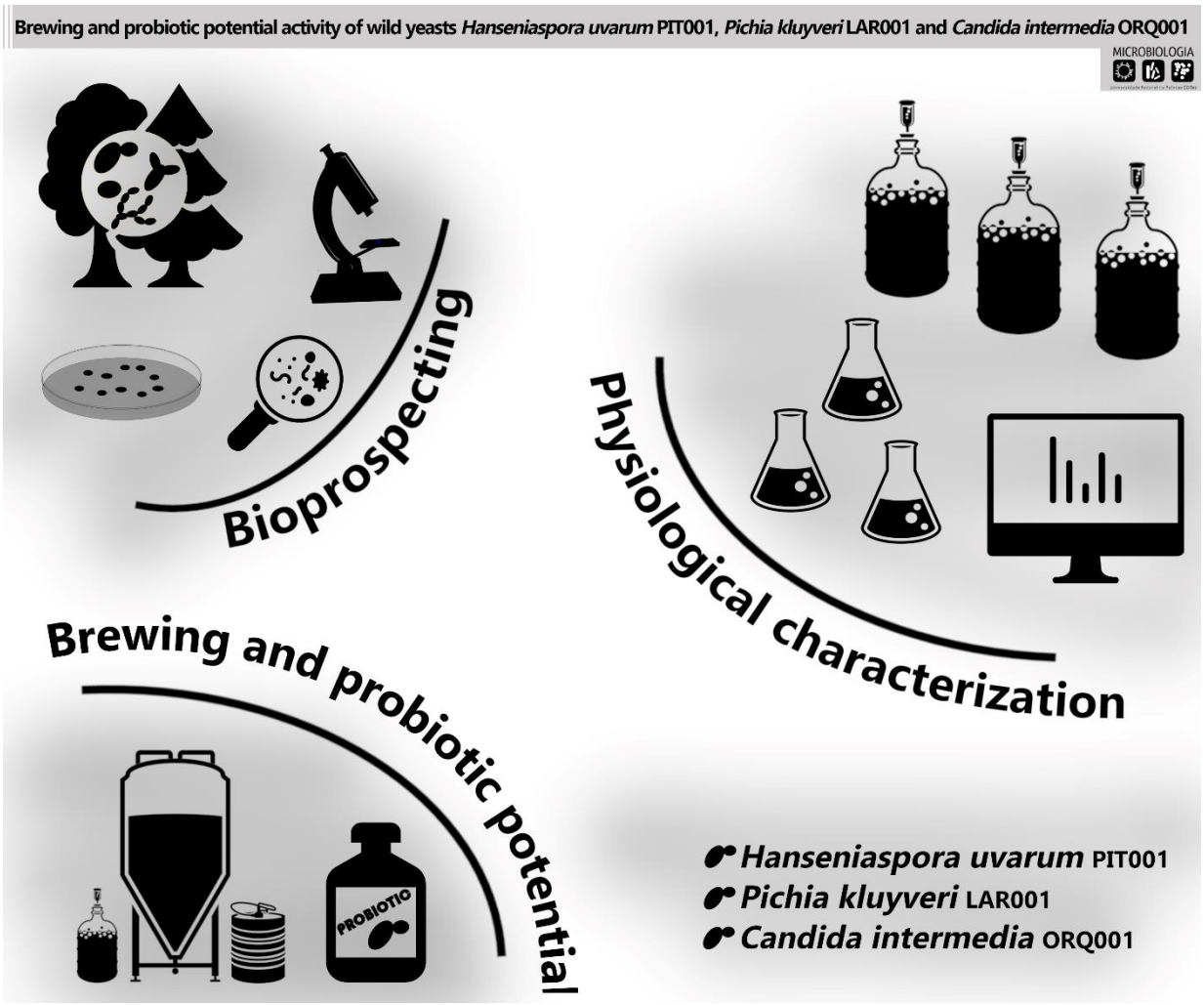
Graphical Abstract



Abstract

Non-conventional yeasts can be isolated from a wide range of environmental sources, often found in beverage industry in mixed fermentations, in which the microorganisms' inoculum usually is not fully known. It is important to know starter cultures, since in addition to favoring reproducibility, other properties can be discovered. Thus, the objective of this work was to identify and characterize yeasts isolated from environment, evaluating their probiotic potential and possible use in brewery. Isolates were obtained from flowers, fruits,

leaves and mixed-fermentation beers, being identified by PCR. Yeasts with promising activity were evaluated regarding their growth under different pHs, temperature and presence of organic acids. To explore probiotic potential, *in vitro* tests were performed of antimicrobial activity and co-aggregation with food pathogens, auto-aggregation, and survival in simulated gastrointestinal tract conditions. In our study, *Pichia kluyveri* (LAR001), *Hanseniaspora uvarum* (PIT001) and *Candida intermedia* (ORQ001) were selected among 20 isolates. *P. kluyveri* was the only one that tolerated pH 2.5. Lactic acid was not inhibitory, while acetic acid and incubation at 37 °C had a partially inhibitory effect on yeasts growth. All yeasts tolerated α-acids from hops and NaCl up to 1%. It is suggested that isolates are able to adhere to intestinal cells and influence positively the organism in combating pathogens, as they showed auto-aggregation rates above 99% and antagonistic activity to pathogenic bacteria. The yeasts tolerated gastric environment conditions, however were more sensitive to pancreatic conditions. We conclude that isolated non-conventional yeasts showed probiotic potential and promising application in beer fermentation.

Keywords: beer, *Candida intermedia*, *Hanseniaspora uvarum*, non-conventional, *Pichia kluyveri*, probiotic potential, yeasts

Declarations

Funding

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Conflicts of interest

The authors declare no competing interests

Availability of data and material

All data generated or analysed during this study are included in this published article.

Code availability

Not applicable

Author's contributions

All authors contributed to the study conception and design. Material preparation was performed by Renan Eugênio Araujo Piraine and Gustavo Maas Retzlaf. Data collection and analysis were performed by Renan

Eugênio Araujo Piraine. The first draft of the manuscript was written by Renan Eugênio Araujo Piraine and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Ethics approval

Not applicable

Consent to participate

Not applicable

Consent for publication

Not applicable

Introduction

Yeasts are ubiquitous in the environment, being often isolated from the microbiota of fruits, plant exudates, soil and insects (Tikka et al., 2013). Non-conventional yeasts, also named non-*Saccharomyces*, represent an interesting alternative for industry in the development of new products (Steensels and Verstrepen, 2014; Holt et al. 2018). Yeasts like *Brettanomyces* spp., *Candida* spp. and *Pichia* spp. were considered only contaminants in brewing environments, however they are currently considered important components in high-value added beers (Michel et al., 2016). These fermenting microorganisms are often found in open fermentations (or exposed to environment), a process which can be unpredictable and can generate large economic losses to breweries (Steensels and Verstrepen, 2014). In the brewing industry, applying pure cultures in fermentations tends to be the first choice, however it is observed in other industries (wine, fermented dairy products, among others) that addition of multiple strains in a controlled manner is a common practice. Mixed fermentations make possible to create products with unique aromatic *bouquets* and distinct nutritional characteristics, obtained according to the concentration of strains used in fermentation process (Holt et al., 2018).

Among wild yeasts, *Hanseniaspora* spp. have been described as predominant on some fruits surface (especially grapes) and are known for significantly contribute in the sensory profile of different wine styles (Martin et al. 2018). Also, strains from other genera like *Pichia* spp. and *Candida* spp. have already been used by researchers and breweries to produce beers with low or no alcohol content, representing an important application which can be protected by patents (Bellut et al. 2018). Non-*Saccharomyces* yeasts are constantly explored for their bioflavoring ability in fermented beverages, such as beer, wine and cider (Gutiérrez et al. 2018; Holt et al. 2018). This exploration revealed that strains of *P. kluyveri* are capable to enhance levels of acetate esters, contributing with fruitiness in beer and improving quality of wines (Holt et al. 2018; Vicente et al. 2021). Strains of *H. uvarum* have been characterized specially for wine production, however when applied in co-fermentations with *S. cerevisiae* for beer fermentation *H. uvarum* was able to significantly impact in glycerol and acetic acid levels, contributing with complexity and aroma intensity (Matraxia et al. 2021). *C. intermedia* strains have been identified in contaminated beers and other beverages (e.g. juices, dairy products) (Pham et al. 2011; Corbett and de Smidt 2019), however there are no reports of fermented beverages using *C. intermedia* in a controlled way, therefore its contribution to beer flavors is still unknown. Metabolic aspects regarding the use of different substrates, growth at different temperatures and pHs, halotolerance, osmotolerance and enzymatic activity must be well characterized, then industrial application of these isolates can be determined as viable (Cassanego et al. 2017).

Some yeasts have the ability to produce antimicrobial compounds, capable of inhibiting the growth of pathogenic bacteria and other fungi (Younis et al. 2017). This characteristic and others are important to confer the classification as "probiotics", which by definition are live microorganisms that, when administered in adequate amounts, confer benefits to host's health (FAO, WHO, 2001). To be considered probiotics, these microorganisms need to have some functional properties such as: not being pathogenic, resisting harsh conditions found on human gastrointestinal (GI) tract, cell adhesion capacity, having immunostimulatory action, among others (Bevilacqua et al. 2009; Fakruddin et al. 2017). Probiotic potential was described for certain strains of *P. kluyveri*, *H. uvarum* and *C. intermedia*, involving their ability to produce antimicrobial proteins (Labbani et al. 2015; Younis et al. 2017), growth repression of pathogens (Goerges et al. 2006; Tiago et al. 2009), auto-aggregation and co-aggregation with pathogens (Ogunremi et al. 2015; Yildiran et al. 2019),

and tolerance to gastrointestinal conditions (Ogunremi et al. 2015; Cassanego et al. 2017). However, the probiotic potential varies among strains, what highlights the importance of characterizing them through several *in vitro* and *in vivo* tests.

Saccharomyces cerevisiae and *S. boulardii* are two yeasts that stand out for their probiotic activity. The search for non-*Saccharomyces* yeasts with probiotic potential arises from different sources, such as plant and animal fermented foods as well as wild fermented beverages (Amorim et al., 2018; Cassanego et al., 2017; Zivkovic et al., 2014). Depending on the isolate, different yeast species may have as much probiotic potential as those of *Saccharomyces* genus. Thus, the objective of this work was to isolate wild yeasts, identifying and characterizing their fermentation capacity, sensory contribution for beer worts and their probiotic potential.

Material and methods

Wild yeasts isolation

Using a sterile swab soaked in YM medium (0.3% yeast extract, 0.3% malt extract, 0.5% bacteriological peptone and 1% glucose) there was a friction on surface of the fruits strawberry (*Fragaria* ^{x} *ananassa*), blackberry (*Morus nigra*), cherry (*Eugenia uniflora*), orange (*Citrus* ^{x} *sinensis*) and butiá (*Butia capitata*), being subsequently inoculated in a tube containing YM added with ampicillin (100 µg/µL). Cultures were incubated for 48 h at 28 °C with constant agitation of 150 rpm. For flowers and leaves of orchid (*Aspasia lunata*), pitaya (*Hylocereus undatus*), vine (*Vitis vinífera*), and *Red Flanders* and *Old Ale* beers, the same procedure was performed. All samples collected for yeasts isolation were obtained in the city of Pelotas (Latitude -31.776, Longitude -52.3594 31° 46’ 34” South, 52° 21’ 34” West), throughout spring/summer period, with daily temperatures around 25 to 30 °C.

Yeasts were isolated by streaking samples from cultures on YM agar + ampicillin. Plates were kept at 28 °C for 72 h for yeasts growth. The yeast morphology was observed using optical microscope BLUE1600BA-L-BT (Biofocus, Brazil) at 1000x magnification. Isolates were stored at Microbiology Laboratory Yeast Bank (Federal University of Pelotas, Brazil) by freezing (- 80°C) using glycerol 30%, with identification codes being given to each isolate.

Fermentation of synthetic beer must

In order to evaluate fermentation ability in standard beer worts, a synthetic beer must was formulated with 400 mL of malt extract (Dry Brew, Liotecnica, Brazil) with a density of 11 °P, pH 5.0, sterilized by autoclaving for 15 min at 121 °C was used. Iso-α-acid from hops (Hopsteiner, Germany) was added to final 15 IBU (International Bitterness Units). It was standardized a concentration of 10^9 yeast cells (total) to ferment 400 mL of synthetic beer must, thus all isolates were cultivated in YM medium over 48 h, 28 °C and 150 rpm condition prior fermentation test, and yeast cells concentration was obtained by counting in a Neubauer chamber. A volume corresponding to 10^9 cells in each yeast culture was decanted by centrifugation at 1.500 × g for 5 min in DTR-16000 centrifuge (DAIKI, Korea), then cell pellet was suspended with 15 mL of the synthetic beer must, subsequently re-inoculated in the total volume for fermentation.

Fermentation was conducted during 14 days, in an incubator at 22 °C, with no agitation. Production and release of CO_2 was monitored through bubble formation in an air-lock piece, while biofilm formation, on medium surface, was visually observed. After this period, final density and pH were evaluated using a pHmeter (KASVI, Brazil), a densimeter (Incoterm, Brazil) for density in g/cm³ measure and a refractometer (AKSO, Brazil) for measure in Brix degrees, data used to calculate percentage of apparent attenuation by each isolate. Apparent attenuation (AA) was calculated using Beersmith™ v.3 software (Beersmith, United States), following the formula: **AA% = 100 × (OG-FG)/(OG-1.000)**, in which "OG" means Original Gravity and "FG" is Final Gravity, both being used in g/cm³. Aroma resulting after the 14 day fermentation period was evaluated by simple sensory analysis consisting of multiple individuals, as conducted in Osburn *et al.* (2016) study. *Saccharomyces cerevisiae* YT001 (Yeastech, Brazil) was used in this experiment as a reference yeast, as well as a baseline for our sensory analyses.

DNA extraction, PCR and sequencing

Total DNA from each isolate was extracted following the protocol described by Preiss et al. (2018) and its concentration quantified in Nanovue™ (Biochrom, United States). Identification at species level was performed using ITS1 and ITS4 primers, which are responsible for amplifying the repetitive region of the 5.8S rRNA gene and ITS flanking regions (Internal Transcribed Spacer). PCR reaction was performed using 0,7 uL of previously extracted DNAs (1 ug/uL), 22 uL of Master Mix (Ludwig Biotecnology, Brazil), 1 uL (0.5 uM) of ITS1 primer (5′TCCGTAGGTGAACCTTGCGG) and 1 uL (0,5 uM) of ITS4 primer (5′TCCTCCGCTTATTGATATGC), for a total volume of 24,7 uL in the reaction. Following incubation conditions for PCR were used: initial denaturation at 95 °C for 10 min, 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 2 min and extension at 72 °C for 2 min, and then final extension at 72 °C for 8 min. PCR products sizes were analyzed by electrophoresis of 10 uL in 1% agarose gel (w/v) in TBE buffer (Tris 0.89 M, EDTA 0.02 M, Boric Acid 0.89 M), under 100 V, 500 mA over 2 h. Agarose gels were stained with 5 uL of ethidium bromide and bands sizes were predicted by comparison with 1 kb Plus DNA Ladder (ThermoFischer Scientific, United States).

The PCR product of each isolate was purified using GFX PCR DNA and Gel Band Purification (GE Healthcare, United States), quantified with Nanovue™ and then submitted at an approximate concentration of 50 ng/l uL for sequencing by ACTGene Análises Moleculares company (Brazil) through Applied Biosystems AB-3500 platform. Data regarding PCR products sequencing were used for alignment using BlastN tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and the NCBI (National Center for Biotechnology Information) data base for yeast identification.

Characterization of yeasts isolates

Evaluation of yeast growth in different stress conditions

Wild yeasts *Hanseniaspora uvarum* (PIT001), *Pichia kluyveri* (LAR001) and *Candida intermedia* (ORQ001) were selected in addition to *S. boulardii*, a yeast which was already part of microorganism bank of Microbiology Laboratory in the Technological Development Center of the Federal University of Pelotas.

Cultivation tests were performed in 10 mL of YPD medium (1% yeast extract, 2% bacteriological peptone, 2% glucose), following a protocol adapted from Zeng et al. (2019). Analysis of resistance to different conditions were tested: lactic acid 1%, acetic acid 0.2% and pHs 2.5, 6.0 and 8.0. Yeast cultures were maintained for 72 h at 28 °C, in an orbital shaker at 150 rpm. In addition, yeast growth was evaluated in YPD pH 6.0 at 37 °C. Samples were collected at 0 h, 4 h, 24 h, 48 h and 72 h, analyzing their biomasses by absorbance reads at 600nm in Biochrom Ultrospec-10 spectrophotometer (Amersham Biosciences, United States) and CFU/mL (Colony Forming Units). Viable cells (CFU/mL) were counted by serial dilutions using saline solution (0,9% NaCl) and plating in agar YPD medium, following a 48 h incubation period at 28 °C. Growth index (GI) was calculated based in Zeng et al. (2019) and Bevilacqua et al. (2009) method, following the equation: GI= AbSs/AbSc × 100, where AbSs is the absorbance of the samples in different pH, temperature and organic acids presence, and AbSc is the absorbance of the control samples. Values < 25% were considered high inhibitory activity, between 25% - 75% moderate one and > 75% growth was weak activity.

Selected yeasts were also cultivated in YPD with different concentrations of Sodium Chloride (NaCl) salt, ranging from 0.5%, 1%, 5% and 10% (p/v), to assess the ability to tolerate ionic stress by halotolerance. Yeast growth was evaluated after 48 h at 28 °C by optical density, and if $OD_{600nm} \geq 1.0$ (representing $\geq 1 \times 10^7$ cells), it was considered that yeast isolate was able to survive and grow in this condition.

Sensitivity to α-acids (spot test)

The analysis of yeast tolerance to α-acids from hops was performed based on adapted protocol from Samanfar et al. (2017). The tolerance of isolated yeasts to 200 ppm of isomerized hop extract 30% (Hopsteiner, Germany) was evaluated in agar YM medium, in which were applied 10 µL of 10X dilutions of yeast cultures in initial concentration of 10^8 CFU/mL. Plates were incubated for 48 h at 28 °C.

Determination of proteolytic activity

Protease activity was evaluated following protocol established by Zeng et al. (2019). Briefly, skim milk agar medium was used (1% skim milk, 0.1% glucose, 0.5% bacteriological peptone, 0.25% yeast extract, 1.5% agar), in which 10 µL from yeast cultures at 10^8 CFU/mL were applied. Positive result should be observed with formation of a halo around the drop, after an incubation during 48 h at 28 °C.

Gelatinase test

Gelatinase activity was evaluated according to Pereira et al. (2009) protocol. Culture medium for gelatinase test was prepared with 1% yeast extract, 1.5% bacteriological peptone and 12% gelatin. The isolates were previously cultured in YPD medium during 24 h at 28 °C, then transferred to the medium containing gelatin with a platinum loop. Tubes were incubated at 28 °C for 7 days, and after that were maintained under refrigeration (4 °C – 10 °C) for 30 minutes. Positivity (i.e. enzymatic activity) in the test

was based on the conversion of semi-solid medium into liquid. *Staphylococcus aureus* ATCC 25923 was previously cultivated in Brain Heart Infusion (BHI) medium and then used as a positive control in the gelatinase test.

Auto-aggregation and co-aggregation

Auto-aggregation and co-aggregation tests were performed following Collado et al. (2008) protocols. Briefly, yeasts were cultivated for 24 h at 28 °C in YM medium, centrifuged for 5 min at 2.000 × g and absorbance OD_{600nm} adjusted to 0.25 ± 0.02 using Phosphate-Buffered Saline (PBS). Auto-aggregation test was conducted under 18 °C, 28 °c and 37 °C. Absorbance at 600nm was read at 2h and 20 h and these values were used in the equation [1-A_{600nm} of final suspension/A_{600nm} of initial suspension) x 100] for results as percentage.

In the co-aggregation test, pathogens *Escherichia coli* ATCC8739 (Gram-negative bacterium) and *Listeria monocytogenes* ATCC 7644 (Gram-positive bacterium) were cultivated for 24 h at 37 °C in BHI medium, centrifuged during 5 min at 8.000 × g, then their absorbance at 600nm were adjusted to 0.25 ± 0.02 using PBS solution. Equal volumes of pathogen and yeast were mixed (1:1) and incubated under the same conditions as performed in auto-aggregation test, as well as absorbance reads times. Results for co-aggregation were expressed as percentage, originated from the equation $[(A_{pat} + A_{isol}) – (A_{mix})/(A_{pat} + A_{isol})]$ x 100, in which "$A_{pat} + A_{isol}$" represents the absorbance value for pathogen + isolate in time 0 h and "A_{mix}" is the absorbance of microorganisms' mixed suspensions in different periods of time.

Antimicrobial activity test

Bacteria related to foodborne diseases (FBDs) *Pseudomonas aeruginosa* ATCC27853, *L. monocytogenes* ATCC 7644, *S. aureus* ATCC25923, *E. coli* ATCC8739 and a wild isolate of *Klebsiella* sp. were used to test the capacity of isolated yeasts to inhibit growth of bacterial pathogens. Antimicrobial activity test was carried out according to the double-layer technique protocol presented by Amorim et al. (2018), with values of inhibition halos being expressed in millimeters (halo + drop).

Gastrointestinal tract *in vitro* simulation

Gastrointestinal tract simulation tests were performed based on protocol described by Bonatsou et al. (2015), with some adaptations. Simulation of GI tract conditions was performed using two solutions: Gastric Digestion (GD) and Pancreatic Digestion (PD). Gastric digestion solution was prepared with NaCl (2.05 g/L), KH_2PO_4 (0.60 g/L), $CaCl_2$ (0.11 g/L), KCl (0.37 g/L), pH 2.0 adjusted with HCl 1 M and autoclaved for 15 min at 121 °C; after sterilization, pepsin (0.0133 g/L) and lysozyme (0.01 g/L) were added. Pancreatic digestion solution was prepared with bile salts (3.0 g/L), Na_2HPO_4 (26,9 g/L), NaCl (8,5 g/L), pH 8.0 adjusted with HCl 1 M; after sterilization, pancreatin enzyme (0.1 g/L) was added. Yeasts were submitted to simulations of GD and PD, with solutions applied separately and in sequence.

Initially, 1 colony was selected from fresh cultures streaked on YPD agar to be inoculated in 10 mL of YPD medium, with incubation at 28 °C until reaching 10^8 CFU/mL concentration. After collecting a sample, yeast cultures were centrifuged at 1.500 × g for 10 min, washed with GD solution, and then suspended in 10 mL of the same solution. Incubation was carried out at 37 °C for 2.5 h, under agitation at 200 rpm in order to simulate peristaltic movements, and at test ending a sample was collected. Then cultures were centrifuged again at 1.500 × g for 10 min, washed with PD solution, and suspended in 10 mL of the same solution. Yeast cultures remained at 37 °C for 3.5 h, under the same agitation condition as described in previous test. Viable yeast cells were detected according to yeast counts before and after each treatment.

Concentrations of viable yeast cells were used to determine viability index (VI) during the test, based on the equation proposed by Zeng et al. (2019): VI=$log\ N_t/log\ N_0$ x 100, in which N_t is related to yeast concentration at specific time and N_0 refers to the initial cell concentration. The test was also performed in an isolated manner, in which it was split in two independent tests, one with GD solution and another one with PD solution, aiming to obtain specific VI for PD step. Again, yeasts started these tests with a concentration of 10^8 CFU/mL.

Statistical analysis

Data were analyzed by analysis of variance (ANOVA) and Tukey's test to determine significant difference ($p < 0.05$) between means. All statistical analyzes were performed using GraphPad Prism 7 software.

Results

Wild yeast isolation and identification

Morphological characteristics of colonies and cells, as well as the respective source of isolation are shown in table 1. PCR-amplified products were used in Blast N tool, which enabled the identification of 20 wild yeast isolates, with 9 different species (>99% similarity and identity).

Table 1: Identification of wild yeast isolates. Description of macromorphological aspects of colonies and cells observed through microscopy. It was possible to identify isolates at species level through PCR using ITS1 and ITS4 primers, with subsequent sequencing of PCR-amplified product (AP).

Isolate	Isolation source	Colony morphology	Cell morphology	Species	AP*
AMO001	Blackberry (*Morus nigra*)	Circular, flat, entire margin	Ellipsoidal, rarely ovoid	*Hanseniospora uvarum*	750 pb
AMO003	Blackberry (*Morus nigra*)	Circular, flat, entire margin	Ellipsoidal, rarely ovoid	*Hanseniospora uvarum*	750 pb
ORQ001	Orchid (*Aspasia luneta*)	Circular, flat, entire margin	Ovoid	*Candida intermedia*	420 pb

ORQ002	Orchid (*Aspasia luneta*)	Circular, flat, entire margin	Ovoid	*Candida intermedia*	420 pb
PAR001	Vine (*Vitis vinífera*)	Circular, flat, entire margin	Ellipsoidal, rarely ovoid	*Hanseniospora uvarum*	750 pb
PAR002	Vine (*Vitis vinífera*)	Circular, flat, entire margin	Ellipsoidal, rarely ovoid	*Hanseniospora uvarum*	750 pb
PIF001CR	Pitaya (*Hylocereus undatus*)	Circular, raised, ondulated margin	Ovoid, rarely ovoid	*Candida manassasensis*	650 pb
PIF2.001	Pitaya (*Hylocereus undatus*)	Circular, raised, ondulated margin	Ovoid, rarely ovoid	*Candida manassasensis*	650 pb
PITS002	Cherry (*Eugenia uniflora*)	Circular, raised, entire margin	Ovoid	*Saccharomyces cerevisiae*	880 pb
PIT003	Cherry (*Eugenia uniflora*)	Circular, flat, entire margin	Ellipsoidal, rarely ovoid	*Hanseniospora uvarum*	750 pb
PIT004	Cherry (*Eugenia uniflora*)	Circular, flat, entire margin	Ellipsoidal, rarely ovoid	*Hanseniospora uvarum*	750 pb
MOR001	Strawberry (*Fragaria x ananassa*)	Circular, flat, entire margin	Ellipsoidal, rarely ovoid	*Hanseniospora uvarum*	750 pb
MOR003	Strawberry (*Fragaria x ananassa*)	Circular, flat, ondulated margin	Elongated	*Issatchenkia terricola*	450 pb
COQ001	Jelly palm fruit (*Butia capitata*)	Circular, brillant, raised, entire margin	Ovoid	*Cryptococcus terrestris*	510 pb
LAR001	Orange (*Citrus x sinensis*)	Irregular, flat, filiform margin	Ovoid, rarely ellipsoidal	*Pichia kluyveri*	450 pb
REF003	Red Flanders beer (mixed fermentation)	Circular, flat, entire margin	Ovoid	*Pichia manshurica*	500 pb
REF005	Red Flanders beer (mixed fermentation)	Circular, flat, entire margin	Ovoid	*Pichia manshurica*	500 pb
JRO001	Old Ale beer (mixed fermentation)	Circular, raised, filiform margin	Elongated or ovoid, sometimes forming chains	*Pichia kludriavzezii*	350 pb
CAR001	Old Ale beer (mixed fermentation)	Circular, raised, filiform margin	Elongated or ovoid, sometimes forming chains	*Pichia kludriavzezii*	350 pb
AMB001	Old Ale beer (mixed fermentation)	Circular, raised, filiform margin	Elongated or ovoid, sometimes forming chains	*Pichia kludriavzezii*	350 pb
SBO	Yeast bank of Microbiology Laboratory	Circular, raised, entire margin	Ovoid	*Saccharomyces boulardii*	850 pb
YT001	Yeast bank of Yeastech Laboratory	Circular, raised, entire margin	Ovoid or spherical	*Saccharomyces cerevisiae*	880 bp

Fermentation test

We observed that *H. uvarum* isolates maintain as apparent attenuation between 5% - 18%, with emphasis on *H. uvarum* PIT001, responsible for the lower final pH and the highest apparent attenuation (18%) among *H. uvarum* isolates (table 2). Isolates classified as composing *Candida* genus showed important differences in aromatic profile, with *C. intermedia* producing aromas similar to wood, floral, spice and clove-like, while *C. manassasensis* contributed with citrus and tropical sensory notes. For both *C. terrestris* and *P. manshurica* yeasts, no apparent attenuation was observed, as well as a production of aromatic notes that stood out. *P. kludriavzezii* (also known as *Issatchenkia orientalis*) showed an apparent attenuation of 9%, with strong aromatic notes referring to solvent, phenolic, grape and green apple. *P. kluyveri* isolate presented 5% of apparent attenuation, with moderate acidification and an aromatic profile similar to commercial strains of *S. cerevisiae*, being possible to note esters referring to banana.

The *S. cerevisiae* isolate PITS002 was the only one identified as wild *Saccharomyces* yeast. This isolate showed an apparent attenuation level above 50% (indicating the consumption of sugars other than

just glucose), existence of a foam (or *krausen*) at the liquid surface due to intense fermentation, no biofilm formation, and the production of aromas with pleasant fruity notes and a variety of esters, mostly banana, normally detected in higher levels in beers produced by specific *S. cerevisiae* strains (Schneiderbanger et al. 2016; Holt et al. 2019). It was noted for *I. terricola* a low apparent attenuation (5%), however remarkable sensory notes referring to *funky* were detected, descripted as barnyard and horse blanket-like.

Table 2: Results of synthetic beer must fermentation. Fermentation profile and sensory contribution were obtained after 14 days of fermentation at 22 °C. All yeast isolates were inoculated at the same cell concentration (10^9 cells/total).

Isolate	Species	Apparent attenuation	Final pH	Biofilm	Sensory notes (aroma)
AMO001	*Hanseniospora uvarum*	9%	4.46	-	Grapes, wine, floral, sour
AMO003	*Hanseniospora uvarum*	9%	4.41	-	Grapes, wine, floral, sour
ORQ001	*Candida intermedia*	5%	4.40	+/-	Wood, floral, spice, clove
ORQ002	*Candida intermedia*	9%	4.49	+/-	Wood, floral, spice, clove
PAR001	*Hanseniospora uvarum*	9%	4.40	-	Grapes, wine, floral, sour
PAR002	*Hanseniospora uvarum*	5%	4.41	-	Grapes, wine, floral, sour
PIF001CR	*Candida manassasensis*	5%	4.58	+	Citrusy and tropical notes
PIF2.001	*Candida manassasensis*	14%	4.28	+	Citrusy and tropical notes
PITS002	*Saccharomyces cerevisiae*	50%	4.13	-	Fruity, ester, banana
PIT001	*Hanseniospora uvarum*	18%	4.00	-	Grapes, wine, floral, sour
PIT004	*Hanseniospora uvarum*	14%	4.39	-	Grapes, wine, floral, sour
MOR001	*Hanseniospora uvarum*	9%	4.40	-	Grapes, wine, floral, sour
MOR003	*Issatchenkia terricola*	5%	4.38	+	*Funky*, phenolic, barnyard, horse blanket
COQ001	*Cryptococcus terrestris*	0%	4.35	-	Neutral
LAR001	*Pichia kluyveri*	5%	4.35	+/-	Slightly esterified, banana
REF003	*Pichia manshurica*	0%	4.59	-	Neutral
REF005	*Pichia manshurica*	0%	4.51	-	Neutral
JRO001	*Pichia kludriavzezii*	9%	4.43	+	Solvent, phenolic, grapes, green apple
CAR001	*Pichia kludriavzezii*	9%	4.44	+	Solvent, phenolic, grapes, green apple
AMB001	*Pichia kludriavzezii*	9%	4.47	+	Solvent, phenolic, grapes, green apple
SBO	*Saccharomyces boulardii*	32%	3.8	-	Slightly esterified, alcoholic, spicy, clover
YT001	*Saccharomyces cerevisiae*	55%	3.95	-	Neutral

Yeasts culture under different stress conditions

Growth index (GI)

The isolates identified as *P. kluyveri* (LAR001), *H. uvarum* (PIT001) and *C. intermedia* (ORQ001) were selected for further characterization tests, based in the ability of certain strains of these yeasts to participate in co-fermentations of beers, bioflavoring potential and antimicrobial properties. These isolates and the control *S. boulardii* were cultured and evaluated under different stress conditions, in which biomasses produced were analyzed by absorbance at OD_{600nm}. For GI determination, only final absorbance was evaluated. Observing growth index (Table 3), it was possible to observe that yeasts presented growth similar to the control (GI > 75%) when incubated in YPD pH 8.0, it representing a weak or no inhibitory

condition for yeasts growth. Low pH was a high inhibitory condition to *H. uvarum* and *C. intermedia* development (GI < 25%), causing to these yeasts not to increase their biomass over time when cultivated at pH 2.5; it was detected that *S. boulardii* culture was also impacted in this condition, it causing a partial growth inhibition of this strain (GI 25% - 75%). We observed that *P. kluyveri* adapted itself and tolerated both media with pH 2.5 and 8.0, since its biomass had significant increase already at the beginning of the cultivation, reaching OD_{600nm} greater than 5.

Lactic acid at 1% concentration was not inhibitory to any yeast, what was demonstrated on growth index values. Yeast cultivation in YPD medium containing 0.2% acetic acid revealed that *P. kluyveri* and *H. uvarum* were not able to overcome biomass above OD_{600nm} 3.2 throughout the test, demonstrating this was a condition with partial growth inhibition (GI 25% - 75%). *C. intermedia* was the only yeast that failed to multiply its biomass, which was a high inhibitory condition for this yeast. As observed in GI for 37 °C condition, this temperature was inhibitory for *H. uvarum* and *C. intermedia* growth, and partially inhibitory for *P. kluyveri*.

Table 3: Growth index of yeasts culture under different growing conditions. GI values < 25% were considered high inhibitory activity, between 25% - 75% moderate one and > 75%growth was weak activity. G.I. = Growth Index. OD600nm = final absorbance of cultures after 72 h of cultivation.

	pH 2.5		**pH 8.0**		**Lactic acid 1%**		**Acetic acid 0.2%**		**37 °C**		**Control**
	O.D.$_{600nm}$	**G.I.**	**O.D.$_{600nm}$**	**G.I.**	**O.D.$_{600nm}$**	**G.I.**	**O.D.$_{600nm}$**	**G.I.**	**O.D.$_{600nm}$**	**G.I.**	**O.D.$_{600nm}$**
P. kluyveri	5.15	> 75	6.50	> 75	5.60	> 75	3.05	25 - 75	4.40	25 - 75	6.50
H. uvarum	0.04	< 25	4.90	> 75	4.65	> 75	3.15	25 - 75	0.14	< 25	4.90
C. intermedia	0.03	< 25	6.65	> 75	9.00	> 75	0.10	< 25	1.79	< 25	8.35
S. boulardii	4.35	25 - 75	6.65	> 75	6.00	> 75	5.20	25 - 75	5.70	> 75	7.25

Cell viability evaluation

When yeasts were incubated in the condition considered optimal for their growth (YPD pH 6.0, incubation temperature 28 °C), it was observed a same behavior for all yeasts: they started the test (4 h) with 10^5 CFU/mL, increased their growth to 10^8 CFU/mL in 24 h and 48 h time points and then ending (72 h) with 10^7 CFU/mL (Fig. 1).

C. intermedia and *H. uvarum* presented respectively 10^4 CFU/mL and 10^5 CFU/mL at the beginning (4h) of YPD pH 2.5 test, however due to inability to withstand this hostile environment, no live cells were found in the next time points. Both *P. kluyveri* and *S. boulardii* already had 10^5 CFU/mL at the beginning of their cultivation, with a viability peak being reached at 48 h (10^7 CFU/mL). However, after 72 h *P. kluyveri* presented a decrease of 1 $\log_{10}$ in CFU/mL concentration, ending the test with 10^6 CFU/mL.

During YPD pH 8.0 test, only *P. kluyveri* started (4h) with 10^6 CFU/mL, while all other yeasts presented 10^5 CFU/mL. *P. kluyveri*, *C. intermedia* and *H. uvarum* showed the highest live cell concentration at 24 h time point (10^8 CFU/mL), maintaining same concentration at 48 h for *C. intermedia* and *H. uvarum* or decreasing to 10^7 CFU/mL in *P. kluyveri* culture.

Responses of *P. kluyveri* and *H. uvarum* to acetic acid-induced stress were similar, where both yeasts reached 10^4 CFU/mL at 24 h of culture and then remained at 10^6 CFU/mL in 48 h and 72 h time points. *C. intermedia* started the test with 10^4 CFU/mL (4 h) and no longer showed capacity to increase live cell concentration, revealing its difficulty in resisting acetic acid stress. Lactic acid presence in culture medium revealed that although *P. kluyveri* and *H. uvarum* reached a concentration of 10^7 CFU/mL in 48 h time point, after 72 h their cultures had a decrease of 1 $\log_{10}$ in cell concentration, totaling 10^6 CFU/mL for both. The yeast *C. intermedia* was the only one that maintained a prolonged period of adaptation in this condition, between 0 h – 24 h (10^4 CFU/mL), and then keeping growing until the end of the experiment, presenting 10^8 CFU/mL at 72 h time point.

Experiment conducted at 37 °C revealed that *C. intermedia* was able to grow up to 10^6 CFU/mL in 24 h, *P. kluyveri* and *S. boulardii* up to 10^7 CFU/mL and only *H. uvarum* demonstrated the inability to tolerate this harsh condition, representing a limiting factor for its growth. Concentration of 10^7 CFU/mL after 72 h was observed only for *S. boulardii*, while *P. kluyveri* and *C. intermedia* remained at 10^6 CFU/mL at the experiment ending.

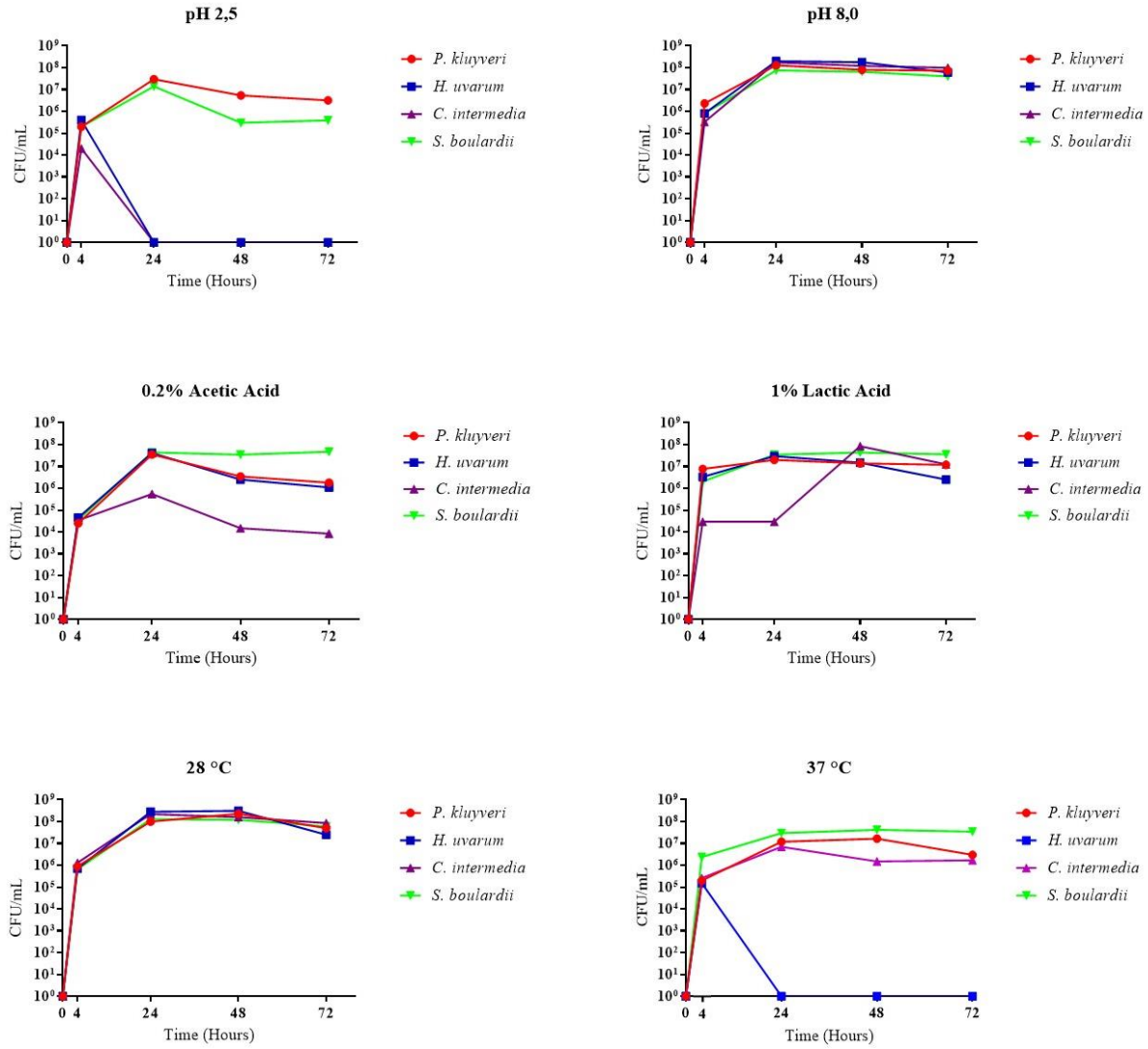

Fig. 1 Evaluation of cell viability of *P. kluyveri* (LAR001), *H. uvarum* (PIT001), *C. intermedia* (ORQ001) and *S. boulardii* cultivated in YPD under different conditions. Graphs show CFU/mL concentration for the four yeasts incubated with YPD media at pH 2.5 and 8.0, lactic acid 1%, acetic acid 0.2% and at a temperature of 37 °C. Yeast cultivation in YPD medium pH 6.0, incubated at 28 °C was considered as optimal condition for yeast growth, representing a control culture. Total experiment time, time points for samples collection and agitation were maintained the same in all tests. Graphs were created in GraphPad Prism 7 software

Tolerance to α-acids from hops

In the analysis of yeasts tolerance to hop α-acids we observed that 200 ppm of this compound was tolerated by the three isolates, as well as *S. boulardii*, a yeast genotipically close to *S. cerevisiae* and with similar biological behavior. The *S. cerevisiae* tolerance to this α-acids concentration was previous demonstrated in Piraine et al. (2021b). Figure 2 shows different dilutions of yeasts culture applied in solid culture medium containing isomerized hop extract, from which we could determine that *P. kluyveri*, *H. uvarum* and *C. intermedia* were resistant to high concentrations of this compound.

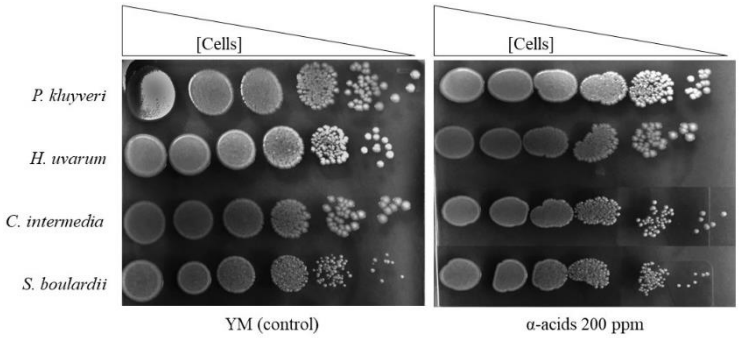

Fig. 2 Evaluating the sensitivity of *P. kluyveri*, *H. uvarum* and *C. intermedia* to α-acids from hop extract. There were no differences visualized between cells grown on YM (control) and YM medium containing 200 ppm of α-acids, evidencing tolerance by them. Culture dilutions were made in 10x, from 10^8 UFC/mL samples

Halotolerance

Regarding salinity (ionic) tolerance, all yeasts were able to grow up to a concentration of NaCl 1%, however only *P. kluyveri* and *C. intermedia* are able to tolerate the ionic stress created by NaCl at a concentration of 5%. Also, at the highest concentration tested (NaCl 10%) only *C. intermedia* was able to maintain its growth, obtaining $O.D._{600nm} > 1.0$ (as shown in table 4).

Table 4: Tolerance of yeasts to different concentrations of NaCl in YPD media. Concentrations of NaCl ranging between 0.5% to 10% were added to YPD media, aiming to characterize halotolerance in isolated yeasts. A plus sign (+) represents cell growth above $O.D._{600nm}$ 1.0, while minus sign (-) indicates there was no growth in the culture tested.

	NaCl			
	0,5%	**1%**	**5%**	**10%**
P. kluyveri	+	+	+	-
H. uvarum	+	+	-	-
C. intermedia	+	+	+	+
S. boulardii	+	+	-	-

Auto-aggregation and co-aggregation

It was possible to observe auto-aggregation characteristic was affected when at 18 °C, in which all isolates presented a lower percentage of auto-aggregation (< 90%) than those observed at 28 °C and 37 °C ($p < 0.05$). Yeasts showed high auto-aggregation capacity within 20 h (> 98%) under temperatures of incubation between 28 to 37 °C, with no statistical differences among the three isolates (table 5).

Co-aggregation analysis with *E. coli* (table 6) and *L. monocytogenes* (table 7) revealed temperature also influenced on percentage of aggregation, it being less than 60% for all yeasts at 18 °C. Values for co-aggregation increase when evaluated at temperatures of 28 °C and 37 °C, remaining between 65 – 70% after 20 h at 37 °C for all isolates. It could be also observed in these two temperatures that co-aggregation

percentages are higher when yeasts are mixed with *L. monocytogenes*, increasing the rates between 5 – 12% when compared to *E. coli* ($p < 0.05$).

Table 5: Auto-aggregation of *P. kluyveri*, *H. uvarum* and *C. intermedia* at different time points and temperatures. Results refers to two independent tests, performed in duplicate. Different lowercase letters within the same column indicate significant differences according to Tukey's test ($p < 0.05$), comparing isolates to each other at the same temperature and incubation period. Different uppercase letters in the line were used to indicate significant difference ($p < 0.05$) in auto-aggregation percentages in different incubation temperatures, for each isolate at the same incubation period.

	Auto-aggregation (%)					
	18 °C		**28 °C**		**37 °C**	
	2h	**20h**	**2h**	**20h**	**2h**	**20h**
P. kluyveri	16.04 ±0.08 ^{bC}	88.22 ±0.46 ^{bB}	43.80 ±0.54 ^{aA}	98.25 ±1.70 ^{aA}	26.10 ±4.90 ^{bB}	99.88 ±0.12 ^{aA}
H. uvarum	9.02 ±0.12 ^{dB}	84.25 ±0.64 ^{cB}	20.00 ±0.24 ^{bA}	98.57 ±1.38 ^{aA}	25.62 ±6.82 ^{bA}	99.92 ±0.08 ^{aA}
C. intermedia	13.51 ±1.91 ^{cC}	83.75 ±0.50 ^{cB}	23.93 ±0.09 ^{cA}	99.95 ± 0.04 ^{aA}	18.12 ±1.65 ^{bB}	99.87 ±0.06 ^{aA}
S. boulardii	22.02 ±1.63 ^{aB}	95.87 ±0.25 ^{aA}	38.05 ±1.63 ^{dA}	99.87 ±0.07 ^{aA}	42.25 ±4.92 ^{aA}	99. 83 ±0.16 ^{aA}

Table 6: Co-aggregation of *P. kluyveri*, *H. uvarum*, *C. intermedia* with *E. coli* ATCC8739 at different time points and temperatures. Results refers to two independent tests, performed in duplicate. Different lowercase letters within the same column indicate significant differences according to Tukey's test ($p < 0.05$), comparing isolates to each other at the same temperature and incubation period. Different uppercase letters in the line were used to indicate significant difference ($p < 0.05$) in co-aggregation percentages in different incubation temperatures, for each isolate at the same incubation period.

	Co-aggregation *E. coli* ATCC 8739 (%)					
	18 °C		**28 °C**		**37 °C**	
	2h	**20h**	**2h**	**20h**	**2h**	**20h**
P. kluyveri	16.06 ±3.16 ^{aA}	58.03 ±1.63 ^{aB}	11.98 ±0.02 ^{bB}	64.02 ±0.05 ^{bA}	12.07 ±0.15 ^{bB}	66.02 ±1.63 ^{bA}
H. uvarum	14.07 ±1.64 ^{aA}	50.15 ±1.66 ^{bC}	10.00 ±1.63 ^{cB}	60.30 ±0.47 ^{cB}	14.00 ±1.59 ^{bA}	68.11 ±0.08 ^{bA}
C. intermedia	8.12 ±3.27 ^{bA}	58.10 ±1.82 ^{aB}	0.18 ± 0.13 ^{dB}	68.08 ±0.17 ^{aA}	4.15 ±0.30 ^{cC}	66.13 ±1.65 ^{bA}
S. boulardii	18.11 ±1.61 ^{aB}	57.98 ±1.50 ^{aC}	20.08 ±0.17 ^{aAB}	63.81 ±0.16 ^{bB}	22.10 ±1.53 ^{aA}	72.12 ±0.11 ^{aA}

Table 7: Co-aggregation of *P. kluyveri, H. uvarum, C. intermedia* with *L. monocytogenes* ATCC7644 in different time points and temperatures. Results refers to two independent tests, performed in duplicate. Different lowercase letters within the same column indicate significant differences according to Tukey's test ($p < 0.05$), comparing isolates to each other at the same temperature and incubation period. Different uppercase letters in the line were used to indicate significant difference ($p < 0.05$) in co-aggregation percentages in different incubation temperatures, for each isolate at the same incubation period.

	Co-aggregation *L. monocytogenes* ATCC 7644 (%)					
	18 °C		**28 °C**		**37 °C**	
	2h	**20h**	**2h**	**20h**	**2h**	**20h**
P. kluyveri	2.14 ±1.64 ^{abC}	54.05 ±1.55 ^{aB}	20. 01 ±0.02 ^{bB}	70.03 ±1.53 ^{bA}	26.10 ±1.61 ^{bA}	73.25 ±2.21 ^{cA}
H. uvarum	1.62 ±1.09 ^{abB}	50.12 ±1.84 ^{bC}	20.04 ±0.04 ^{bA}	68.00 ±0.01 ^{bB}	22.00 ±1.63 ^{cA}	76.00 ±0.01 ^{acA}
C. intermedia	0.27 ±0.20 ^{bB}	52.01 ±0.02 ^{abC}	0.36 ±0.30 ^{cB}	70.12 ±1.65 ^{bB}	12.12 ±0.25 ^{dA}	78.02 ±1.63 ^{bA}
S. boulardii	4.06 ±0.11 ^{aC}	54.03 ±1.63 ^{aC}	26.06 ±1.57 ^{aB}	74.13 ±1.60 ^{aB}	32.23 ±0.25 ^{aA}	78.60 ±1.95 ^{aA}

Enzymatic activity and antagonistic effect against pathogens

The test performed to analyze enzyme activity showed that none of the yeasts had positivity for gelatinase, thus all of them were considered negative for its activity. During yeast characterization, proteolytic activity was also evaluated, and the negative reaction was found again for all isolates.

We observed all yeasts were able to inhibit bacterial growth in different degrees. We could highlight the inhibition performed by *P. kluyveri* for *L. monocytogenes* and by *H. uvarum* for *S. aureus*, in which a high capacity of inhibition was detected. For other food contaminants, for example *P. aeruginosa*, it was also found high inhibitory activity being performed by isolates, even having similar results to those observed for probiotic *S. boulardii* (Fig. 3).

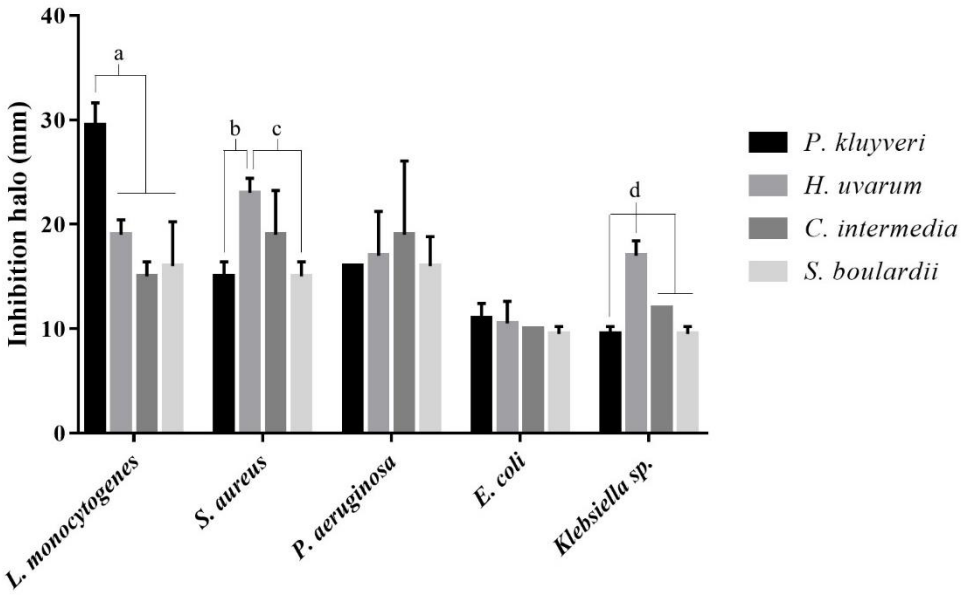

Fig. 3 Antimicrobial activity by *P. kluyveri*, *H. uvarum*, *C. intermedia* and *S. boulardii* against pathogen bacteria related to FBD. Bacterial growth inhibition was detected through visualization of halo formation, which represents antimicrobial activity by isolated yeasts. Statistical difference was established by analysis of variance ANOVA ($p < 0.05$), in which groups with differences are indicated in the graph by letters: **a**: *P. kluyveri* was different from other groups; **b**: *P. kluyveri* differs from *H. uvarum*; **c**: *H. uvarum* was different from *S. boulardii*; **d**: *H. uvarum* differs from the other yeasts

Yeasts tolerance to *in vitro* GI tract simulated conditions

Isolated yeasts were evaluated regarding their survival after submission to GD and PD solutions, what simulated a complete trajectory in GI tract. Figure 4a presents live cells concentration (CFU/mL) before and after yeasts incubation in GD solution. There was no statistical difference ($p < 0.05$) for any yeast after GD passage, demonstrating that although a harsh condition, it was not enough to significantly decrease cell viability count.

We observed viable cell count before and after pancreatic digestion solution, in which *H. uvarum* and *C. intermedia* showed a decrease from 10^8 CFU/mL to 10^7 CFU/mL (1 $\log_{10}$ decrease), while for *P. kluyveri* it was observed a final concentration of 10^6 CFU/mL (2 $\log_{10}$ decrease). Data collected could also be analyzed by viability index (Fig. 4b), in which all isolates presented VI ≥ 93% for GD solution. Meanwhile, after PD solution, these rates decreased to 84% for *H. uvarum*, 88% for *C. intermedia*, and 75% for *P. kluyveri.*

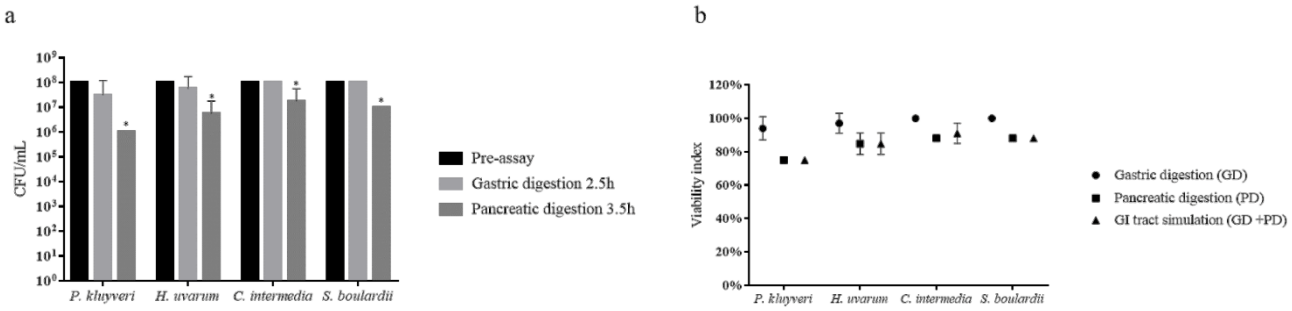

Fig. 4 Cell viability analysis before and after incubation in GI tract simulated conditions. A) Yeast tolerance was evaluated under adverse conditions found in GI tract, with incubation of yeast cells on gastric digestion (GD) and later in pancreatic digestion (PD) solution. No statistical difference was observed between cell viability before and after GD solution, for all yeasts cultures. After incubation under PD conditions, a decrease in viable cell concentration was observed for *H. uvarum*, *C. intermedia* and *P. kluyveri.* Statistical difference in data was evaluated by analysis of variance ANOVA ($p < 0.05$). * indicates significant difference in cell viability during the test. B) Viability Index (VI) of yeasts under GD, PD and GD + PD conditions: all yeasts showed VI ≥ 93% for GD solution, while a decrease to 88% after PD solution (tested in isolated method) could be observed for *H. uvarum* and *C. intermedia*. Since it was observed a decrease of 2 $\log_{10}$ in *P. kluyveri* cell count, a VI = 75% was determined for PD and GD + PD test

Discussion

Wild yeasts are easily found in sugar-rich sources (Tikka et al. 2013), the challenge is to isolate them from existing microbiota and to explore their potential for use in industry. Malt extract and beer worts

are composed of maltose, and to a lesser extent glucose, maltotriose, fructose and sucrose (Hansen and Wasdovitch 2005). Some yeasts are not able to ferment maltose, so their attenuation profile is reduced to fermentation of simple sugars. However, yeasts with a low percentage of attenuation in beer wort contribute significantly to their flavors (Michel et al., 2016), being also used in the production of other fermented beverages (e.g. wine, spirits, mead) or even they can be used in the formulation of beers with low or no alcohol content. We observed through preliminary sensory analysis that isolates had the ability to produce flavors like floral, fruity, phenolic, among others. In this aspect, *P. kluyveri* can be highlighted, which in its fermentation presented a remarkable esterified banana aroma, similar to flavors described by Saerens and Swiegers (2017). Even though very often non-*Saccharomyces* yeasts are not responsible for high attenuation percentages, these yeasts show potential to be used in sequential or co-fermentations with more attenuative yeasts (generally *Saccharomyces*), as already reported by Holt et al. (2018), Lu et al. (2017), Matraxia et al. (2021) and Michel et al. (2016), enhancing flavor complexity of beers, wines, and others.

Three yeasts were selected from all isolates to perform characterization tests: *P. kluyveri* (LAR001), based on its probiotic potential and production of low-alcohol beers (Fai et al. 2014; Saerens and Swiegers 2017), *H. uvarum* (PIT001), for its aromatic profile, contributions to the fermentation of beverages (Martin et al. 2018) and GRAS status (Generally Regarded as Safe) (López et al. 2016) and *C. intermedia*, for their distinct flavors and potential antimicrobial activity (Younis et al. 2017). Characterize wild isolates permits to explore their physiological characteristics, tolerance under stressful conditions, secondary metabolites produced, technological performances, and predict suitability for use as starters in fermentation processes (Steensels and Verstrepen 2014; Michel et al. 2016b, a). Proteolytic activity is usually analyzed because it is related to specific flavors production in fermented products, like meat (Zeng et al. 2013), however not all microorganisms have this activity as observed in our study and in Zeng et al. (2019). Properties like ability to withstand different pH ranges are of great importance, for sour beer production (Rogers et al. 2016) and mainly to characterize yeasts regarding their tolerance to GI tract conditions. Media formulated with pH 6.0 favors yeast growth because it is a normally tolerable acidity (Murakami et al. 2011), configuring it as a standard pH in many culture media for these microorganisms. In YPD pH 8.0 test, all yeast cultures had the same cell concentration as observed at pH 6.0 after 72 h and GI index > 75%, what indicates the adaptability to basic pH without significant impacts in their cell viability. It was observed not all yeasts were able to maintain cell multiplication in different pH ranges, as noted for *C. intermedia* and *H. uvarum*, which did not tolerated pH 2.5. Analyzing cell viability at pH 2.5, it was observed for *P. kluyveri* 1 $\log_{10}$ less in cell concentration value than that observed in other pHs, what could be related to physiological disturbances caused by extremely low pH, such as loss of minerals to the extracellular environment, decreased in trehalose levels and cell aging (Murakami et al. 2011; Reis et al. 2013).

Yeasts tolerance to stress caused by increase in incubation temperature is well described for several *S. cerevisiae* strains (Munna et al., 2015), however data for non-*Saccharomyces* yeasts are still reduced to a few studies. In our work, incubation temperature of 37 °C was shown to be a limiting growth condition for *H. uvarum*, and partially inhibiting *C. intermedia*. Steensels and Verstrepen (2014) reported for cocoa fermentations and mixed fermentations beers, when temperature remains around 30 °C, *Hanseniaspora* spp. is among the first microorganisms to multiply, however following fermentation process with

temperatures above 35 °C, *Pichia* spp., *Candida* spp. and *Saccharomyces* spp. are main yeasts acting in fermentation. These data corroborate with our findings, in which *P. kluyveri* and *C. intermedia* sustained their growth in 37 °C incubation test. Survival and tolerance of yeasts to 37 °C incubation are also important to know their probiotic potential, as yeasts will be submitted to this temperature during transit in GI tract (Czerucka et al., 2007).

Lactic acid bacteria and acetic acid bacteria compose the microbiota present in GI tract and are able to produce significant amounts of organic acids, which can affect viability of yeasts cells (Zeng et al. 2019). Narendranath et al. (2001) noted in minimal media supplemented with lactic acid or acetic acid, that lactic acid concentrations of 0,8%-1% reduced yeast growth rate sharply, and lower concentrations of acetic acid (0.05%-0.1%) had the same effect. Based on Narendranath et al. (2001) and Zeng et al. (2019) experiments, we tested moderate levels of these organic acids to evaluate acid tolerance by the isolated yeasts. We observed that lactic acid presence did not significantly impact final biomasses of any yeasts tested; meanwhile, acetic acid impacted both biomass and viable cell concentration of all yeasts.

Tolerance to ionic stress (halotolerance) is an important characteristic when exploring new starter cultures, since some fermented foods such as meat, olives and "salted" beers demand yeasts in their process that resist this condition (Bevilacqua et al., 2009). Thus, it was observed that all isolates were able to grow in concentrations up to 1% of NaCl, while only *P. kluyveri* and *C. intermedia* had same ability in YPD with NaCl 5% added. As described in Bevilacqua et al. (2009) and Stratford et al. (2019) in which were highlighted some species of *Candida* spp. regarding its tolerance to NaCl, we observed in our study *C. intermedia* was the only one able to grow in YPD with NaCl 10%. When applied in beer fermentations, it is interesting that yeasts are tolerant to hop α-acids (one of the main compounds with antimicrobial properties), since it can inhibit their growth (Osburn et al., 2018). In the hop tolerance test, it was observed yeasts having similar growth to the control (YM medium without iso- α-acids), what suggested resistance by yeasts at high concentrations (200 ppm) of the compound. Michel et al. (2016a) and Methner et al. (2019) have demonstrated that hop compounds can have a negative effect on yeasts growth (even restricting their application in conventional beers like IPAs), however we observed a sufficient iso-α-acids tolerance in all analyzed yeasts, suggesting their application as viable in a wide range of extremely hopped beers.

Microorganism auto-aggregation is a necessary property for adhesion to intestinal epithelial cells, in which rates above 80% are considered good auto-aggregators (Syal and Vohra, 2013). In our study all isolates showed values higher than 80% after 20 h of assay, even being above 99% at the warmer temperatures. Co-aggregation is an alternative mechanism to inhibit pathogenic bacteria growth in human intestine (Zeng et al. 2019). *P. kluyveri, H. uvarum* and *C. intermedia* demonstrated co-aggregation values above 50% in co-aggregation tests, however it was observed that temperature was a key factor during the process. It was also possible to identify in 28 °C and 37 °C incubation temperatures yeasts showed greater co-aggregation values for *L. monocytogenes* than *E. coli* ($p < 0.05$), regardless the isolate. Antagonistic activity is one of the main properties desired in a probiotic, because represents the ability to hinder or even prevent the development of pathogens that penetrate through organism mucosal sites (Amorim et al. 2018). The ability to inhibit bacterial pathogens growth by isolated yeasts was similar to observed for *S. boulardii*, and for some bacteria even superior, what demonstrates the probiotic potential of these yeasts. This characteristic is also important for the control of contaminants during beer fermentation, since some

bacteria of *Escherichia, Klebsiella, Pseudomonas,* and *Staphylococcus* genera are often detected in spoiled beers or participating in mixed-fermentations of sour beers (Ashtavinayak and Elizabeth 2016; Piraine et al. 2021a).

The production of gelatinase by probiotics is generally analyzed for being related to safety in its use, because pathogenic microorganisms usually produce this enzyme as part of their pathogenesis (Syal and Vohra, 2013). As observed by Syal and Vohra (2013) and Fakruddin et al. (2017) in wild yeast isolates, gelatinase enzyme activity was not found in our isolates, it being a preliminary indication of its safety.

Several microorganisms demonstrate their probiotic potential through pathogens inhibition, however they still need to overcome barriers during passage through GI tract, which involves gastric juice, digestive enzymes, organic acids, bile salts and considerable variations in temperature and pH, such as acidic pH of gastric juice and alkaline pH existing in the intestine (FAO/WHO, 2001; Czerucka et al. 2007). Bonatsou et al. (2015) and Cassanego et al. (2017) demonstrated GD solution is not usually aggressive to yeasts, as also observed in our study; nevertheless, isolates showed to be sensitive to pancreatic conditions, with a decrease of living cells between 1 $\log_{10}$ - 2 $\log_{10}$. In the studies of Cassanego et al. (2017), isolates classified as *S. cerevisiae* and *H. uvarum* were not able to survive after exposure to this condition, however we presented in our work that yeast isolates were able to resist to PD solution. Cell viability analysis during *in vitro* GI tract simulation proves to be important because it is believed that effects related to probiotics are dose-dependent, it being suggested as effective a dosage between 10^7-10^9 CFU/mg per day (Minelli and Benini 2008). Thus, results suggest the concentration of viable cells after GI tract passage is within that expected for probiotic effect in organism.

Yeasts with probiotic activity are being explored in the development of functional beers, i.e. low and non-alcoholic (NA) beers produced with herbs, amino acids, vitamins, minerals, vegetables, or fruits focusing the health benefit and nutritional value for consumers (Habschied et al. 2020). Alcohol-free beers are generally produced by thermal and membrane-based methods, depending of alcohol removal after fermentation by traditional *Saccharomyces* yeasts, or through a biological method, consisting of a controlled mashing and fermentation which limits ethanol formation by the yeast (Bellut et al. 2018; Habschied et al. 2020). These processes can negatively impact beers, modifying aromas, body, and acidity of the final product (Bellut et al. 2018). Alternatively, the isolates of *P. kluyveri*, *H. uvarum* and *C. intermedia* could be employed to produce beers with low levels of ethanol that maintain aromas and desired characteristics, since they are able to ferment only simple sugars and consequently originate less alcohol during fermentation process. However, aiming the development of functional beers, their probiotic potential must be confirmed, requiring more *in vitro* and *in vivo* studies.

Wild yeasts isolate *Pichia kluyveri* (LAR001), *Hanseniaspora uvarum* (PIT001) and *Candida intermedia* (ORQ001) demonstrated probiotic potential, both in relation to inhibition of pathogenic microorganisms and tolerance to harsh conditions of human GI tract. Technological properties regarding application for beer production were also evaluated, demonstrating their contribution on sensory profile and fermentation ability in a synthetic beer must, as well as them as promising candidates for application in other fermentation processes. Future perspectives of this work are *in vivo* tests to confirm their probiotic action and larger scale beer fermentations to stablish them as probiotic starter cultures.

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4.4. **Manuscrito 4 - Immunostimulatory activity of *Pichia kluyveri*, *Hanseniaspora uvarum*, *Candida intermedia*, *Saccharomyces boulardii* and their derivatives on RAW 264.7 macrophages**

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Immunostimulatory activity of *Pichia kluyveri, Hanseniaspora uvarum, Candida intermedia, Saccharomyces boulardii* and their derivatives on RAW 264.7 macrophages

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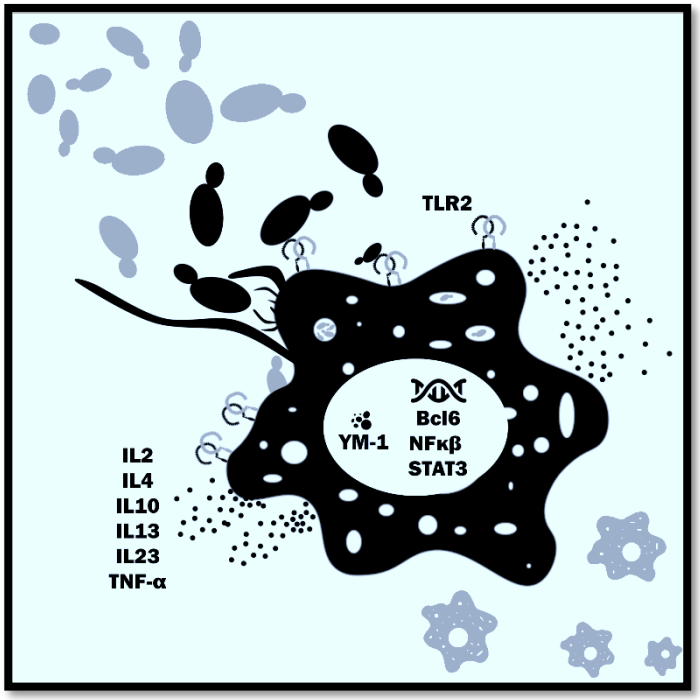
Graphical abstract



Abstract

Yeasts are a group of microorganisms with structural and metabolic characteristics that influence their recognition by immune cells resulting in a species-specific response. Although *Saccharomyces boulardii* is a widely studied probiotic yeast, immunostimulation by non-*Saccharomyces* yeasts still underexplored. Therefore, the aim of this study was to characterize the response induced in macrophages stimulated by yeasts *Pichia kluyveri*, *Hanseniaspora uvarum*, *Candida intermedia* and their derivatives: heat-killed cells, supernatant and DNA. RAW 264.7 murine macrophages were stimulated *in vitro* for 24 h and the response generated was evaluated by analyzing mRNA transcription of cytokines (*IL2*, *IL4*, *IL10*, *IL13*, *IL23*, *TNF-α*), transcription factors (*Bcl6, NFκβ, STAT3*), Toll-like receptor 2 (TLR2) and YM1 protein. Viable and heat-killed cells of *P. kluyveri* and *H. uvarum* were responsible for high levels of relative mRNA transcription of transcription factors and *TLR2* (between 2 – 8-fold increase), however were able to induce

only low transcription levels for analyzed cytokines (≤ 2-fold increase). Viable cells of *C. intermedia* were able to stimulate a significant transcription of *IL4* (7.6-fold increase) and *Bcl6* (4-fold increase), while heat-killed cells stimulated the highest level of *TNF-α* (2.4-fold increase) among yeasts and their derivatives. Furthermore, supernatant from *C. intermedia* culture induced significant ($p < 0.05$) levels of *TLR2* (4.4-fold increase), being the only one among supernatants to present high levels of relative mRNA transcription of *TLR2*. Data found in this work arouse interest in further studies on interaction between non-*Saccharomyces* yeasts and immune system cells, mainly referring to immunomodulatory capacity.

Keywords: Non-*Saccharomyces* yeasts, *C. intermedia*, *H. uvarum*, *P. kluyveri*, macrophage, immunostimulation

Declarations

Funding

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Conflicts of interest

The authors declare no competing interests

Availability of data and material

All data generated or analyzed during this study are included in this published article

Code availability

Not applicable

Ethics approval

Not applicable

Consent to participate

Not applicable

Consent for publication

Not applicable

Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Renan E. A. Piraine, Vitória S. Gonçalves and Neida L. Conrad. The first draft

of the manuscript was written by Renan E. A. Piraine and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Introduction

Microorganisms in the gastrointestinal (GI) tract impact on metabolism, endocrinological system, nervous system GI tract development and immune system regulation [1]. Microbiome present in GI tract is essential for development and function of intestinal mucosa, representing a central barrier in the line of resistance to invasion by pathogens [2]. The microbial community composition is able to prevent and treat bowel disorders (e.g. inflammatory bowel disease), aid in systematic disorders (e.g. allergies) and even enhance the immune response to vaccines [1, 3–5]. Changes in microbial community can be achieved by the administration of probiotic bacteria and yeasts, resulting for example in an immunomodulation in cytokines secretion by immune cells [6].

Gastrointestinal tract is constantly exposed to exogenous substances (bacteria, fungi, peptides, food particles, among others), so 60-70% of the body's immune cells are present in this environment [7]. Macrophages are responsible for initiating response against microorganisms, phagocytizing and identifying them using specific receptors, presenting antigens to other immune cells and participating in the coordination of immune response through the expression of cytokines [8]. Yeasts (microbe-) associated molecular patterns (MAMPs) are recognized by pattern recognition receptors (e.g. TLR2, Dectin-1, among others) on immune cells and by doing so activating or suppressing immune responses [9]. Yeast cell wall components, such as β-glicans and mannans, are recognized by these receptors, inducing specific responses through cytokines and chemokines, which stimulate T cell differentiation [10, 11].

Even though yeasts are part of a same group of microorganisms, they have structural and metabolic differences that influence the stimulation of immune system cells during their recognition, resulting in a variable species-specific response [10]. Probiotic potential of non-*Saccharomyces* yeasts has been widely studied by several groups [12–17], however data regarding the immunomodulation induced by these microorganisms are still scarce on literature. Among non-*Saccharomyces* yeasts, *Hanseniaspora uvarum* (PIT001), *Pichia kluyveri* (LAR001) and *Candida intermedia* (ORQ001) are isolated from our previous studies, which had probiotic characteristics studied *in vitro* (data not shown) and were considered promising yeasts. However, more data are needed regarding their *in vivo* activity and stimulation of effector molecules (cytokines and chemokines), cell receptors and signaling pathways in biological models and cell cultures.

Live yeast cells maintain a microorganism-host interaction through molecules actively produced and secreted, but the simple interaction between non-viable cells (and their cell wall components) with surface receptors expressed by host can be also sufficient to stimulate immune system [6, 18]. Pericolini et al. [19] demonstrate that administration of live or inactivated yeast cells can result in differences in organism's immunomodulation, culminating in different efficacy in controlling pathogens by the host. Yeast secretome is also involved in responses orchestrated by macrophages, as some extracellular proteins present immunogenicity and are related to virulence factors [20]. Proteins secreted by yeasts may also result in changes in cell adhesion and cytokines expression to cell cultures *in vitro* [21]. Nucleic acids from yeasts can be recognized by TLRs (e.g. TLR9) that can signaling for production of interferon (IFN) and other pro-

inflammatory genes expression via Toll/Interleukin-1 receptor (TIR) domain or TIR-domain-containing adaptor inducing IFN-β (TRIF) [22].

Yeast cells, their structures and secreted molecules, are recognized as potential stimulators of immune system, so identify responses induced in immune cells is important to characterize this activity. Thus, in this work we investigated response to stimuli using yeast cells (live and heat-killed), culture supernatant and DNA on RAW 264.7 macrophages, evaluating mRNA transcription of cytokines (*IL2*, *IL4*, *IL10*, *IL13*, *IL23*, *TNF-α*), transcription factors (*Bcl6, NFκβ, STAT3*), Toll-like receptor 2 (TLR2) and YM1 protein.

Material and methods

Strains and culture conditions

Wild isolates *Pichia kluyveri* LAR001, *Hanseniaspora uvarum* PIT001 and *Candida intermedia* ORQ001 were obtained from the microorganism bank of Microbiology Laboratory in the Federal University of Pelotas, as well as the commercial yeast *Saccharomyces boulardii* (a reference probiotic strain), which were cryopreserved in glycerol at -80 °C. Yeasts were grown overnight in YM (Yeast and Malt Extract) medium (0.3% yeast extract, 0.3% malt extract, 0.5% peptone and 1% glucose) at 30 °C under constant agitation of 150 rpm.

Murine macrophage-like cell line RAW 264.7 (ATCC® TIB-71™) was grown as monolayers according to Santos et al. [23]. Briefly, cells were incubated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) Fetal Bovine Serum (FBS) at 37 °C in a 90% humidity atmosphere with 5% CO_2, until approximately 80% confluence in the culture plate.

Stimuli preparation

Live and inactivated (heat-killed) cells, culture supernatants, and DNA extracted from each yeast were used to stimulate RAW 264.7 cell culture. Yeast cells were washed twice with Phosphate Buffered Saline buffer (PBS) and then 10^8 CFU/mL of viable cells were stored to be used subsequently during macrophages stimulus. Cell-free supernatant from yeast culture media was obtained by centrifugation at 2.000 × g for 10 min and then separate in aliquots to further stimulate macrophages. Yeast cells at same concentration (10^8 CFU/mL) were inactivated by heat and pressure (heat-killed cells), autoclaving at 120 °C with a pressure of 1 atm for 20 min. After inactivation, samples were seeded onto YM agar medium and incubated for 48 h at 28 °C, a control step to assure correct yeast inactivation.

Total DNA from yeasts was extracted following an adaptation of the protocol described by Preiss et al. [24]. A volume of 3 mL from yeast cultures was centrifuged in a DAIKI DTR-16000 centrifuge, at 12.000 × g for 1 min, then it was suspended using 200 uL of breaking buffer solution (2% Triton-X 100 w/v, 1% SDS w/v, 100 mM NaCl, 100 mM Tris pH 8.0, EDTA 1 mM pH 8.0). An amount corresponding to 100 uL of glass microbeads (0.5 uM, Sigma-Aldrich) and 200 uL of phenol/chloroform/isoamyl alcohol (25:24:1) were added to previous solution. Tubes were then vortexed for 2 min, adding TE buffer (10 mM

Tris pH 8.0, 1 mM EDTA pH 8.0) after that. Centrifugation was performed at 13.000 × g for 5 min, then aqueous phase (~350 uL) was transferred to a new tube, where 1 mL of ethanol 96% was added, homogenized and incubated at room temperature for 10 min (with no shaking). Tubes were centrifuged at 13.000 × g for 2 min, supernatants were discarded, and pellets were dried at room temperature for 20 min. DNA was eluted with 50 uL of DNAse free water, then DNA quality was evaluated by electrophoresis on 1% agarose gel (100V, 500 mA, 1 h) and its concentration quantified by Nanovue™ (Biochrom).

RAW 264.7 cells stimulation

Stimulation occurred in a yeast:RAW cells ratio of 10:1, following adaptation of the protocol developed by Smith et al. [25]. RAW 264.7 cells were kept under stimulation for 24 h, with incubation in DMEM supplemented with 10% (v/v) FBS at 37 °C in an atmosphere of 90% humidity with 5% CO_2. As negative control, cells were stimulated with DMEM medium only. As positive controls, Concanavalin A – ConcA (10 µg) and Zymosan (100 µg) (Sigma-Aldrich) were used. For the assay, 100 µL of live cells or 100 µL of heat-killed cells per well were used as stimuli, at a final concentration of 10^7 CFU/mL. The same volume of supernatant (100 µL) was used, while for DNA of each yeast the concentration of 850 ng/well was targeted. Total volume for stimulus + culture medium was 1 mL per well, being carried out in triplicates.

RNA extraction, cDNA synthesis and qPCR

RNA was extracted using the TRIzol method, according to the manufacturer's instructions. Reverse transcription was carried out using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) using approximately 400 ng of RNA. The qPCR reactions were performed using 1 µL of cDNA (synthetized from 400 ng RNA), 5 µL of SYBR Green qPCR Master Mix (Promega, USA), 0.25 µL of each primer (from 10 µg/µL solution), and 3.5 µL of RNAse Free Water (Sigma-Aldrich, USA), for a total reaction volume of 10 µL. Conditions of temperature and time for qPCR reaction were performed according de Avila *et al.* (2016) [26], as follows: denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 60 s, extension at 72 °C for 60 s, and a final extension at 72 °C for 5 min.

Relative mRNA transcription levels for *IL2, IL4, IL10, IL13, IL23, TNF-α, Bcl6, NFκB, STAT3, YM1 and TLR2* were determined using qPCR with *β-actin* used as endogenous reference gene. qPCR reactions were performed on a StepOne™ Plus qPCR System (Applied Biosystems). The primers used were described in Table 1. Reaction efficiency for each primer pair was calculated and previously described by [3, 23, 26], and primer specificity was checked from melting curves. All samples were analyzed in triplicate using the comparative threshold cycle (ΔΔCt) method to determine the relative mRNA expression compared to *β-actin* as the reference gene, following a previously described method [27].

Table 1. Sequences of primers used in qPCR reactions.

Gene	Forward (5'-3')	Reverse (5'-3')
β-actin	AGAGGGAAATCGTGCGTGAC	CAATAGTGATGACCTGGCCGT
IL2	TTGTGCTCCTTGTCAACAGC	CTGGGGAGTTTCAGGTTCCT
IL4	CCAAGGTGCTTCGCATATTT	ATCGAAAAGCCCGAAAGAGT
IL10	TTTGAATTCCCTGGGTGAGAA	ACAGGGGAGAAATCGATGACA
IL13	TCTTGCTTGCCTTGGTGGTC	GGTCTTGTGTGATGTTGCTCAGC
IL23	CCTTCTCCGTTCCAAGATCCT	ACTAAGGGCTCAGTCAGAGTTGCT
TNF-α	CTGAGTTCTGCAAAGGGAGAG	CCTCAGGGAAGAATCTGGAAAG
Bcl6	GCCGGCTCAATAATCTCGTGAACAGGTCC	CCAGCAGTATGGAGGCACATCTCTGTATGC
NFκβ	AGTGCAAAGGAAACGCCCAGAAG	GCCAGGGCTTCCGGTACTC
STAT3	CCGATGCCTGTGGGAAGAGTC	TGTCACTACGGCGGCTGTTG
TLR2	ATGTCGTTCAAGGAGGTGCG	CTGACCGGTGATGCAATTCG
YM1	GGGCATACCTTTATCCTGAG	CCACTGAAGTCATCCATGTC

Statistical analysis

Data were analyzed using GraphPad Prism version 7 Software. Differences in mRNA transcription levels were subjected to analysis of variance by two-way ANOVA with multiple comparison, followed by Tukey's test. Differences between groups were statistically significant when $p < 0.05$. Results of the mRNA levels were expressed graphically using fold increase means and standard deviations.

Results

***Saccharomyces boulardii*.** Viable *S. boulardii* cells were able to induce significant ($p < 0.05$) cytokines transcription in RAW cells with 8.6, 8.7, 7.4 and 3-fold increase for *IL2, IL13, IL23, and IL4* respectively (Fig. 1a, d, e and b). Meanwhile for *IL10, TNF-α, YM1, TLR2, Bcl6, NFκβ, STAT3* were detected in low or absent levels (≤ 1.2-fold increase). It was not seen the same response when heat-killed cells were used as stimulus, since relative mRNA transcription for all cytokines remained between 0.5 – 1.2-fold increase (Fig.1). Worth note that transcription factors were induced in higher levels than detected for viable cells, *Bcl6* presenting a 3.3-fold increase transcription, *Nfκβ* and *STAT3* above 1.5-fold increase (1.6 and 1.7-fold, respectively) (Fig. 2a, b and c). Relative mRNA transcription of *TLR2* was low for *S. boulardii* viable cells (0.5-fold increase), however an increase in mRNA levels was observed when its derivatives were used, reaching 1.8-fold increase in heat-killed cells. Supernatant and DNA from *S. boulardii* induced only low levels of mRNA transcription for all genes evaluated, however a remarkable response was observed for *YM1* (2.3-fold increase) in supernatant stimulus (Fig. 3a) and for cytokines *IL2* and *IL10* (1.7-fold increase both) when DNA was used. It was noted that *YM1* mRNA transcription was induced with low (~2-fold increase) levels only in *S. boulardii* supernatant and DNA stimuli, what was not observed for other yeasts and their derivatives (Fig. 3a).

Pichia kluyveri. Live and heat-killed cells of *P. kluyveri* induced a cytokine response close to basal levels, being responsible for low levels in relative mRNA transcription ranging between 0.5 – 1.8-fold increase (Fig. 1). However, these stimuli were able to promote intermediate to high levels of relative mRNA transcription for *Bcl6, Nfκβ* and *STAT3* (Fig.2). Live cells were able to induce a fold increase ranging

between 1.7 – 3.7-fold, while heat-killed cells were able to promote high levels of *Bcl6* (7.3-fold increase) and *STAT3* (3.3-fold increase), with a less extent for *Nfκβ* (2.0-fold increase). A high relative mRNA transcription of *TLR2* (Fig. 3b) was observed for live and heat-killed *P. kluyveri* cells (6.3 and 4.3-fold increase, respectively), suggesting that during yeast recognition there was a potent activation of this receptor. Moreover, *P. kluyveri* cells were able to elicit *YM1* transcription (Fig. 3a), even though at low levels (0.8-fold increase). Supernatant from *P. kluyveri* culture was able to induce low levels of *IL-23* and *TNF-α* (1.3 and 1.4-fold increase), being other cytokines below 1.0-fold in relative mRNA transcription (Fig 1). Similar levels were detected for transcription factors *STAT3* (1.3-fold increase) and *Nfκβ* (1.2-fold increase), however a more prominent response was observed in *Bcl6* induction (2.2-fold increase). Stimulate RAW macrophages with supernatant was also able to induce significant levels of *TLR2*, resulting in 2.2-fold increase in relative mRNA transcription. DNA stimulus promoted only basal levels of mRNA transcription for most genes evaluated, nevertheless it was observed for cytokines *IL4* and *IL10* an increase of 1.6 and 1.8-fold.

Hanseniaspora uvarum. As observed for *P. kluyveri* stimulus, viable cells of *H. uvarum* also resulted in a cytokine response close to basal levels, in which relative mRNA transcription was inferior to 0.7-fold increase for all (Fig. 1). Live cells of *H. uvarum* resulted in significant levels of *Bcl6* transcription, with 3.4-fold increase, the only transcription factor stimulated with significant levels (Fig. 2a). Although live cells were not able to induce significant levels of cytokines transcription, heat-killed cells of *H. uvarum* were responsible for *IL23* transcription with significant levels ($p < 0.05$) when compared to other stimuli derived from this same yeast, resulting in a 2-fold increase (Fig. 1e). Moreover, inactivated cells provoked low to intermediate transcription levels of *IL2* (1.7-fold increase), *IL10* (1.9-fold increase) and *TNF- α* (1.5-fold increase). Observing all transcription factors evaluated (Fig. 2), relative mRNA transcription was higher when heat-killed cells were used ($p < 0.05$), reaching 5.8-fold increase for *Bcl6*, 1.9-fold increase for *Nfκβ* and 3-fold increase for *STAT3*. Transcription levels of *Bcl6* and *STAT3* mRNA after *P. kluyveri* and *H. uvarum* live and heat-killed cells stimuli were superior to those detected for both Concanavalin A and Zymosan ($p < 0.05$). Stimuli with *H. uvarum* were also able to elicit receptor's transcription, with values around 2.3-fold for live and heat-killed cells (Fig. 3b). These values were higher ($p < 0.05$) than those observed for Zymosan (1.4-fold), suggesting these stimuli were also potent TLR2 activators. Supernatant from *H. uvarum* culture was not able to elicit a consistent cytokine transcription (fold increased ranged between 0.29 – 1.0 for most cytokines), being *TNF- α* transcription the most stimulated (1.5-fold increase) and the only one with relative mRNA transcription above 1-fold. The same was observed for other genes tested, in which low or absent levels of mRNA transcription were detected. DNA from *H. uvarum* was responsible for a 2.2-fold increase in relative mRNA transcription of *IL10*, however it was not observed any other significant values for cytokines, transcription factors, *YM1* or *TLR2.*

Candida intermedia. Live cells of *C. intermedia* were shown to be potent activators of *IL4* transcription (7.7-fold increase), with a significant difference from other stimuli also for *IL13* production (2.3-fold increase) ($p < 0.05$) (Fig. 1b and d). Transcription factors, *YM1* and *TLR2* transcription levels were observed at very low levels after stimulus using viable cells (Fig. 3). Macrophage stimulation using inactivated *C. intermedia* cells revealed that higher levels of *TNF-α* were produced (2.4-fold), when compared to other stimuli from yeasts and their derivatives, which were less than 1.5-fold (Fig. 1f). *IL10*

transcription was also observed, however at low levels (1.4-fold increase). Although *Nfκβ* and *STAT3* were detected at low levels (1.5 and 1.6-fold increase, respectively), *Bcl6* relative mRNA transcription was observed at high levels (4.0-fold increase), indicating *C. intermedia* inactivated cells were also *Bcl6* transcription inducers, as detected for *P. kluyveri*, *H. uvarum* and *S. boulardii* (Fig. 2). As observed for previous yeast culture supernatants, it was noted very low levels for most cytokine transcription (≤1.0-fold), except for *IL4* and *TNF-α* which were detected at low levels (1.2 and 1.3-fold increase, respectively). *C. intermedia* culture supernatant also induced a remarkable transcription of *TLR2* (4.4-fold increase) by RAW macrophages (Fig. 3b), evidencing the presence of molecules with ability to stimulate recognition by this receptor. DNA stimulus resulted in a low transcription of *IL4* (1.5-fold increase) and *IL10* (1.7-fold increase), with no significant values for other genes evaluated.

Controls. Zymosan, a polysaccharide isolated from *S. cerevisiae* cell wall and distributed commercially, was used to stimulate macrophages, resulting in consistent levels of transcription of *IL10* and *IL23* (5.5-fold increase for both cytokines) and significant levels of *IL4* and *TNF-α* (3.9-fold increase both). Stimulation by Concanavalin A resulted in a low transcription (1.5 to 2-fold) for all cytokines, except *IL13*, which was observed in concentration practically similar to that of housekeeping gene (basal transcription, 0.2-fold increase only). Zymosan and Concanavalin A stimuli were responsible for low levels of mRNA relevant transcription (ranging 0.5 to 1.5-fold increase) of transcription factors and *TLR2* receptor, however it was seen an intermediate macrophage response in *YM1* for both stimuli (~3-fold increase) (Fig. 4).

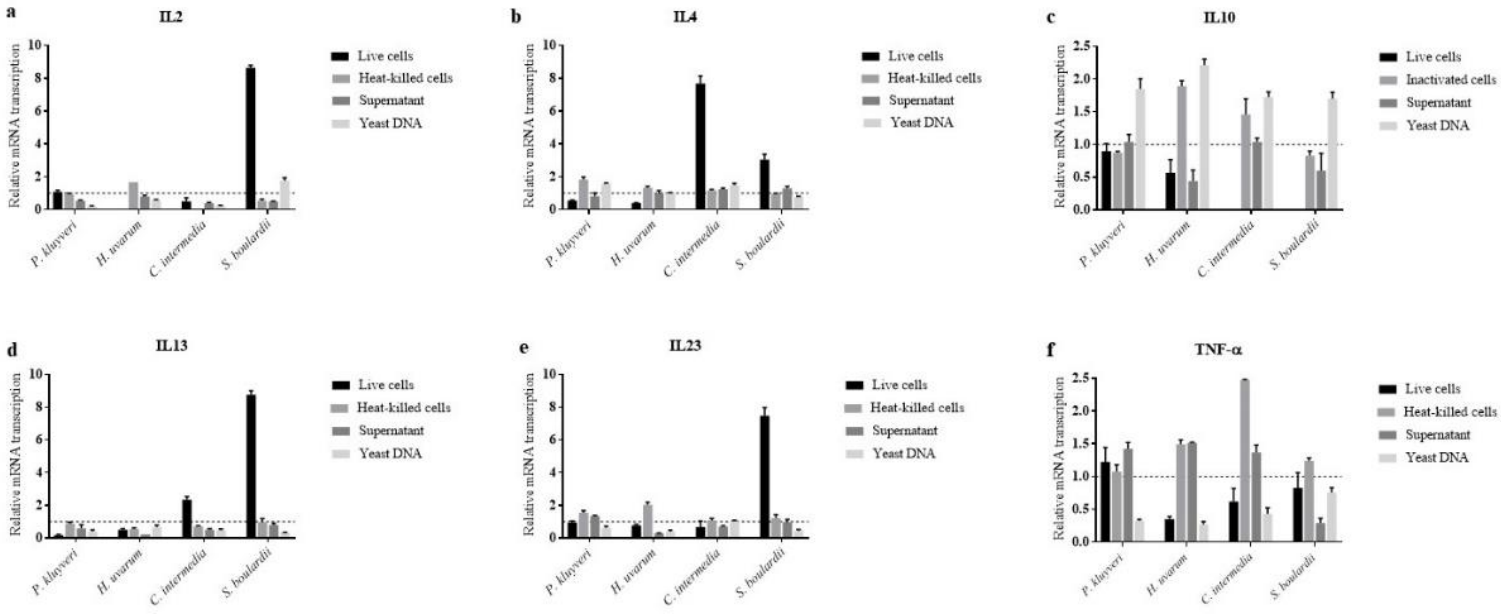

Figure 1: Gene transcription of cytokines during RAW 264.7 stimuli with yeast cells and their derivatives. Viable yeast cells (10^7 UFC/mL), heat-killed inactivated cells (10^7 UFC/mL), culture supernatant (100µl) and fungal DNA (850 ng) from *P. kluyveri* (LAR001), *H. uvarum* (PIT001), *C. intermedia* (ORQ001) and *S. boulardii* (reference probiotic strain) were used to stimulate cytokine secretion by RAW macrophages. Relative mRNA transcription of *IL2* (a), *IL4* (b), *IL10* (c), *IL13* (d), *IL23* (e) and *TNF-α* (f) was normalized using *β-Actin* level as reference. Data are shown as mean ±SD (Standard Deviation). Dotted line indicates one fold of relative mRNA expression.

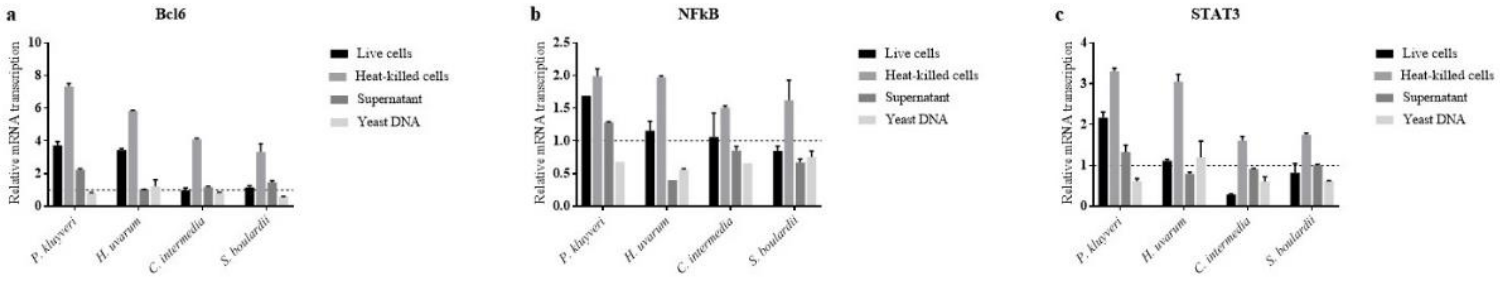

Figure 2: Gene transcription of transcription factors during RAW 264.7 stimuli with yeast cells and their derivatives. Viable yeast cells (10^7 UFC/mL), heat-killed inactivated cells (10^7 UFC/mL), culture supernatant 100µl) and fungal DNA (850 ng) from *P. kluyveri* (LAR001), *H. uvarum* (PIT001), *C. intermedia* (ORQ001) and *S. boulardii* (reference probiotic strain) were used to stimulate transcription factors expression by RAW macrophages *in vitro*. Relative mRNA transcription of *Bcl6* (a), *NFκβ* (b) and *STAT3* (c) was normalized using *β-Actin* level as reference. Data are shown as mean ±SD (Standard Deviation). Dotted line indicates one fold of relative mRNA expression.

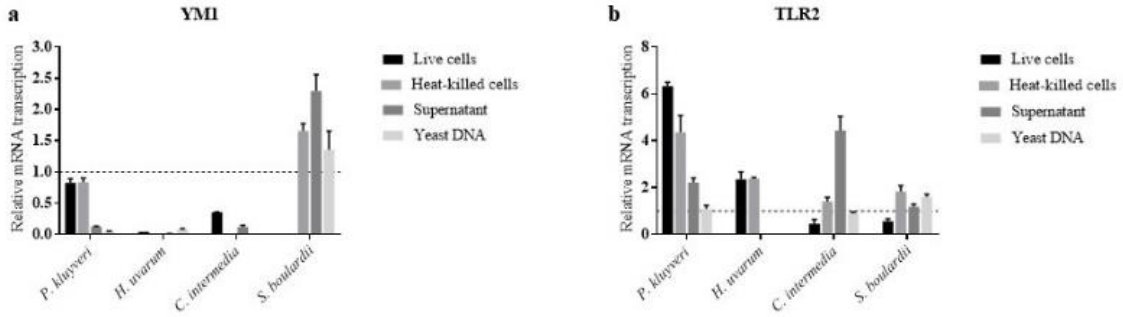

Figure 3: Relative mRNA transcription of YM1 protein and TLR2 receptor during RAW 264.7 stimuli with yeast cells and their derivatives. Viable yeast cells (10^7 UFC/mL), heat-killed inactivated cells (10^7 UFC/mL), culture supernatant 100µl) and fungal DNA (850 ng) from *P. kluyveri* (LAR001), *H. uvarum* (PIT001), *C. intermedia* (ORQ001) and *S. boulardii* (reference probiotic strain) were used to stimulate *YM1* protein and *TLR2* expression by RAW macrophages *in vitro*. Relative mRNA transcription of *YM1* marker (a) and *TLR2* (b) was normalized using β-Actin level as reference. Data are shown as mean ±SD (Standard Deviation). Dotted line indicates one fold of relative mRNA expression.

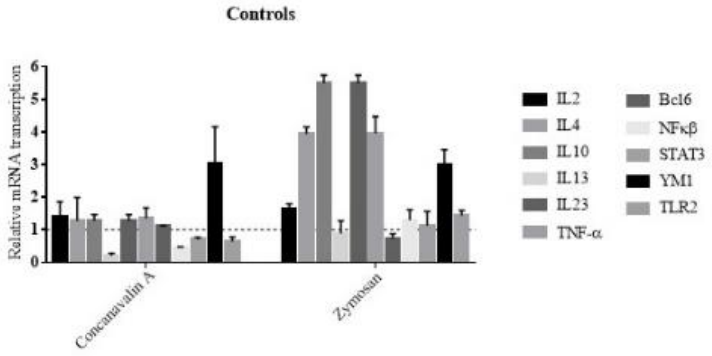

Figure 4: Concanavalin A and Zymosan stimuli in RAW 264.7 macrophage. Data represent relative mRNA transcription of cytokines (*IL2, IL4, IL10, IL13, IL23 and TNF-α*), transcription factors (*Bcl6, NFκβ and STAT3*), *YM1* marker and *TLR2* receptor in RAW macrophages stimulated with Concanavalin A and Zymosan, being determined based on mRNA levels of *β-Actin* reference gene. Error bars indicate ±SD (standard deviation). Dotted line indicates one fold of relative mRNA expression.

Discussion

Yeasts interact with RAW macrophages in different ways

Yeasts, especially those belonging to *Saccharomyces* spp., are able to participate in macrophages activation through stimuli generated when subjected to interaction with these cells [6, 25, 28]. Macrophages when activated can give rise to distinct populations, being M1 and M2 the main populations described. M1 macrophages (or *classically activated*) are polarized *in vitro* by Th1 cytokines such as GM-CSF (Granulocyte-Macrophage Colony-Stimulating Factor), TNF-α and IFN-γ, alone or together with bacterial LPS (lipopolysaccharide). M1 macrophages express pro-inflammatory cytokines such as IL1, IL6, IL12, IL23 and TNF-α. In contrast, M2 macrophages (or *alternatively activated)* are polarized by Th2 cytokines such as IL4 and IL13, producing anti-inflammatory cytokines such as IL10 and TGF-β [8, 29].

Immune response induced by stimulation with yeasts still needs to be explored, as researchers have characterized different response patterns mediated by probiotics, pathogenic yeasts and their components [30, 31]. Analysis of cytokine expression by human DCs (dendritic cells) in Bazan et al. [10] study have verified a response based on IL12, IL23 and IL27 cytokines when stimulated with different yeast genera, including *Saccharomyces* spp. and *Candida* spp. In our study, *Saccharomyces boulardii* was able to stimulate macrophages to produce high transcription levels of *IL2, IL4, IL13* and *IL23*, with low or only basal expression of other cytokines. IL2 promotes growth and development of peripheral immune cells, initiating a defensive immune response through survival and division of regulatory T cells (Treg) and proliferation of cytotoxic T cells [32]. Santos et al. [33] also reported high levels of *IL2* mRNA transcription by PBMCs (Human Peripheral Blood Mononuclear Cells) from animals supplemented with *S. boulardii*. Cytokines IL4 and IL13 play a fundamental role in immunosuppressive and anti-inflammatory activity, inhibiting or decreasing pro-inflammatory cytokines expression such as TNF-α and IL1β [34, 35]. IL23 has an immunoregulatory and pro-inflammatory role, stimulating IFN-γ and mainly sustaining cell-mediated responses focusing in intracellular infections elimination [32, 34]. As noted by Stier and Bischoff (2016) [28] *S. boulardii* leads to a general unspecific immune system activation, and variations in patterns of

cytokines stimulated can be observed according to cell lines (dendritic cells, macrophages, lymphocytes…) analyzed, stimulus, test conditions (*in vitro* X *in vivo*), *in vivo* challenge with pathogens, among others. Although Smith et al. [6] did not find differences in cytokine-inducing properties among live, UV-irradiated and heat-killed cells, in our study it was detected a considerable variation ($p < 0.05$) in relative mRNA transcription between stimuli with viable and non-viable cells, resulting in a 4 to 6-fold less transcription of *IL2*, *IL13* and *IL23* when evaluating heat-killed cells (non-viable cells) of *S. boulardii*.

Hanseniaspora uvarum and *P. kluyveri* are two yeasts which remains underexplored regarding their ability to interact with immune system, so our work is one of the first studies aiming the characterization of immune cells-yeasts interaction. Stimuli using live and inactivated cells from these yeasts maintained a low or basal expression of cytokines evaluated, without a predominance of any of them during cytokine response, as observed for *C. intermedia* and *S. boulardii*. Even so, relative mRNA transcription levels detected were similar to those observed for stimulation with lectin Concanavalin A, a mitogen with well-described activity in macrophage activation [36]. Macrophage response based on *IL4* and *IL13* transcription was observed for live *C. intermedia* cells, with transcription levels for *IL4* 2.5-fold higher than that observed for *S. boulardii*. Variations in metabolic activity and cell wall composition of *Candida* spp. lead to differences in phagocytosis and levels of cytokines being produced by immune cell lines stimulated *in vitro* (such as greater or lesser production of TNF-α) [37] . There are extensive variations in yeast cell wall when comparing different fungal species and strains, such as α-glucans in addition to β-glucans, differences in concentration of chitosan, galactomannans and melanin [11]. Although cell wall composition among *H. uvarum*, *P. kluyveri*, *C. intermedia* and *S. boulardii* is comparable, it was demonstrated by Mateja et al. [38] significant differences in their genera regarding patterns of GPI anchored and non-covalently attached proteins, as well as cell wall thickness, permeability, amounts of mannan and glucans. Thus, different responses induced in immune cells may be related to cell wall complexity and its components [39].

Paraprobiotics are non-viable microorganism cells with capacity to stimulate immune system and modulate its response, amplifying the response through the exposure of immunogenic molecules after an inactivation treatment [18]. After a heat-treatment associated with high pressure, cell inactivation occurs through membranes damage, loss of nutrients and ions, protein denaturation and essential enzymes inactivation, what can lead to modifications in cell coarseness and roughness [18, 40]. These structural and molecular changes influence immune-modulating properties of paraprobiotics, it being reported in some cases even more cytokines production (e.g. IL12) by macrophages stimulated with heat-killed probiotics than viable cells, as observed by Miyazawa [41]. In our study cytokines *IL10*, *IL23* and especially *TNF*-α mRNA transcription levels are potentiated when macrophages are stimulated by heat-killed cells of *C. intermedia* and *H. uvarum*, a behavior also observed for other *Candida* species by Navarro-Arias et al. [37] that stimulated PBMCs with viable and heat-killed *C. albicans*, *C. tropicalis*, *C. guilliermondii*, *C. krusei* and *C. auris*. Based on responses mediated by heat-killed cells, it was suggested also a possible paraprobiotic effect on these yeasts.

In our study, fungal DNA was able to induce a similar pattern of stimuli among all cytokines, however *IL10* was induced at more prominent levels, being the most induced cytokine by these stimuli.

Ramirez-Ortiz et al. [42] and Patin et al. [43] related unmethylated fungal CpG DNA recognition with induction of high levels of TNF-α and other pro-inflammatory cytokines expression, however same relationship was not observed in our experiments. Yeast cells have generated significant transcription levels for cytokines (*IL2*, *IL4*, *IL13* and *IL23* for example), however in some cases DNA stimulus resulted in the highest level of cytokine detected among stimuli, for example in *IL10* transcription, in which all DNAs used generated ≥ 1.5-fold increase in relative mRNA transcription. The conditioning of DCs to the predominance of a response based on IL10 expression promotes Foxp3+ regulatory T cells (Treg) secretion, what contributes to intestinal tolerance [25]. In this context, microorganisms or their derivatives with ability to target this cytokine profile promote tolerance to the intestinal microbiota and are able to reduce conditions characterized by excessive inflammation [7, 25]. It is noteworthy that there is also evidence of IL10 destabilizing cytokines mRNA, including IL10 mRNA itself, so a low cytokine detection in the experiment may be due to this fact [34].

We enforce that our results were obtained from a 24 h period of macrophage stimulation and possibly different responses could be observed if different periods were tested, however it was out of scope in our study. Yeast:immune cells ratio, also known as MOI (*multiplicity of infection)*, is also a parameter that differs among studies, what may influence in patterns of cells interaction and cytokine response [44]. Our methods were in line with Smith et al. [6] and Smith et al. [25], which are important and comprehensive reports of immune cells stimulation by yeasts.

Transcriptional factors role in macrophage stimuli

Analysis of *Bcl6*, *STAT3* and *NFκβ* mRNA expression revealed that heat-killed cells were the stimuli responsible for highest levels of transcription in all tested yeasts. In these cases, *P. kluyveri* and *H. uvarum*, both in viable and non-viable cells, were the yeasts which induced highest levels in expression of transcription factors. Since *Bcl6* plays a fundamental role in regulation of Th2-type inflammation and it is constantly expressed in monocytes [45], we sought to observe in our study its transcriptional levels in stimulated macrophages. *Bcl6* also regulates macrophage function by repressing IL6 pro-inflammatory cytokine production and differentiation of Th17 cells [46]. Observing transcription values for *Bcl6*, a tendency towards M2 polarization of macrophages stimulated with non-*Saccharomyces* yeast cells is suggested, however more studies must to be conducted to confirm this hypothesis.

During active inflammation occasioned by infections, microorganisms can bind to PRRs (Pattern Recognition Receptors) and activate signaling pathways via MAP kinases and *NFκβ*, resulting in stimulation of cytokines production with inflammatory effects, including those with pro-inflammatory activity (e.g. IL1β) which induce polarization to M1 macrophages [47]. The increase in *NFκβ* transcription is involved with TLR2 receptor activity, which has its expression level increased by mannose and β-glicans recognition [48]. Zymosan is a good stimulator of *NFκβ* activation [49], and as observed in our work, stimuli with all yeasts cells (viable or non-viable) were able to induce similar or superior expression of *NFκβ* than observed in Zymosan stimulus.

Toll-like receptors are innate immune response infection sensors that participate in the activation or inhibition of macrophage activity via Jak-STAT pathway; signaling via *STAT3* is activated by several cytokines and their receptors, such as IL2, IL6, IL10, IL23 and IL27 [50]. The STAT proteins are transcription factors frequently involved in downstream cytokine signal transduction mediated by TLR2, TLR4 e TLR9 [51]. *STAT3* main role in macrophages is to mediate anti-inflammatory effects of IL10, restricting gene transcription of pro-inflammatory cytokines [51] and repressively impacting *NFκβ* signaling pathways [52]. *STAT3* transcription was observed at higher levels when heat-killed cells were used as a stimulus, with emphasis on *P. kluyveri* and *H. uvarum,* responsible for inducing significant levels of its expression. These results surpassed induction levels of *Bcl6* and *STAT3* by Concanavalin A and Zymosan by 2 to 6-fold. Therefore, there were indicatives of M2 polarization in macrophages by these stimuli.

Recognition of yeasts and their derivatives by TLR2

Macrophages are usually in an inactive state, but can be activated through a variety of stimuli during immune response, which are recognized in different ways, for example by PRRs such as TLR2 [8, 9, 29, 30, 53]. Yeast cells and their cell wall components (e.g. Zymosan) are important stimulators of TLR2 and Dectin-1 [49, 54, 55], and in our study it was observed that live and inactivated cells of *P. kluyveri* were responsible for high levels of its transcription, up to 4-fold higher than that detected for macrophage stimulation by Zymosan. Stimulus using *H. uvarum* cells also resulted in TLR2 transcription at significant levels, demonstrating this yeast is also responsible for stimulating the receptor expression. Composition of cell walls may vary among yeast species and strains, what modifies the way that immune system recognizes and process their components [54], culminating in higher or lower levels of receptor expression according to yeast surface structure.

Low TLR2 expression from other stimuli does not necessarily impact cytokine expression, as noted by Smith et al. [25], since recognition of cell wall components may also be dependent on other receptors, such as Dectin-1 and mannose receptors. It could also be observed in our data, as although stimuli from *S. boulardii* and its derivatives resulted in low levels of TLR2 expression, cytokines like IL13 were highly expressed when *S. boulardii* live cells were used to stimulate macrophages response. Variations in TLR2 and cytokine expression levels occasioned by live and inactivated cells stimuli may be explained by β-glicans exposure on the entire yeast cell surface occurring after heat treatment, while intact cells usually expose β-glicans only through budding scars [30].

Molecules present on *P. kluyveri* and *C. intermedia* culture supernatants were also responsible for stimulating significant levels of TLR2, higher than observed for Zymosan stimulus, demonstrating metabolites secreted by yeasts are also important in stimulating receptors in cells of the immune system. Secreted proteins by *Candida* spp. yeasts are linked with TLR2/TLR4 recognition as demonstrated by Wang et al. [56], promoting inflammatory response in DCs and macrophages stimulated *in vitro*. The most common ligands related to TLR2 are PAMPs (Pathogen-Associated Molecular Patterns) originated from glycolipids, lipopeptides or GPI-anchored structures [57], thus it is suggested that higher receptor mRNA

transcription in these cases is related to secretome products or proteins detached from yeast cell wall that have these structures in their conformations [21, 56].

YM1 marker

YM1 protein expression is considered a marker for polarization to M2-activated macrophages in mice [58], being initially described in inflammation induced by parasitic infections [59] and observed by our group (data not shown) with relevant expression in M2 macrophages from intestinal mucosa of mice experimentally infected with *Toxocara canis*. Marker expression was not detected for most stimuli, however *P. kluyveri* cells and *S. boulardii* derivatives generated low levels of its transcription. Welch et al. [60] related YM1 expression with IL4 and IL13 production, however this association is questioned by Goren et al. [61], and in our work it was not possible to confirm the same correlation, since live cells of *C. intermedia* and *S. boulardii* (potent activators of these cytokines) had low or absent levels of YM1 transcription.

The highest relative mRNA transcription levels of *YM1* were observed in Concanavalin A and Zymosan stimuli. Concanavalin A showed consistent low levels of mRNA transcription for cytokines, transcription factors and TLR2 on RAW 264.7 macrophages stimulated, however a remarkable response for *YM1* marker could be detected. Zymosan was confirmed as potent *IL10* inducer in immune cells *in vitro* culture, also responsible for a high stimulus of other cytokines, such as *IL4*, *IL23* and *TNF-α*, as well as *YM1* marker.

Conclusion

Saccharomyces boulardii is a yeast that has different probiotic mechanisms already described, mainly immunomodulation in *in vitro* and *in vivo* models. In the present work, the high cytokines transcription levels observed in RAW cells stimulated with *S. boulardii* corroborated with the hypothesis that its probiotic immune modulation mechanism is mediated by cytokines activation. We observed that non-*Saccharomyces* yeasts, *Pichia kluyveri* LAR001, *Hanseniaspora uvarum* PIT001, *Candida intermedia* ORQ001, and their heat-killed cells, culture supernatants and DNAs are able to stimulate RAW macrophages with distinct responses. Viable and heat-killed cells of *P. kluyveri* and *H. uvarum* were responsible for high transcription levels of transcription factors and *TLR2*, but only low levels of relative mRNA transcription for the studied cytokines. Viable cells of *C. intermedia* were able to stimulate significant levels of *IL4* and *Bcl6*, while heat-killed cells stimulated the highest levels of *TNF-α* among yeasts and their derivatives. Furthermore, supernatant from *C. intermedia* culture stimulated high levels of *TLR2*, being the only one among yeast culture supernatants to present high levels of mRNA relevant transcription.

The data found in this work provoke interest in further studies on immunomodulatory activity present in these yeasts. As next steps, we intend to use them in *in vivo* assays to confirm their possible probiotic effects.

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5. Discussão geral e perspectivas

5.1. Metagenoma de cervejas de fermentação mista: leveduras *Saccharomyces* e não-*Saccharomyces*

A utilização de leveduras selvagens em processos fermentativos acompanha o homem há milhares de anos, porém o descobrimento dessa atividade e a participação desses microrganismos foi revelada recentemente (Steensels and Verstrepen 2014). Cervejas Lambic belgas ainda hoje beneficiam-se da microbiota complexa presente em adegas e barris de madeira utilizados em sua produção, cervejas nas quais podem ser observadas diferentes etapas durante a fermentação (com a participação de diferentes bactérias e fungos) de acordo com o tempo e disponibilidade de substratos (Spitaels et al. 2014). A partir da análise de cervejas de fermentação mista de diferentes locais nos Estados Unidos e outros países, observamos que a diversidade de microrganismos é abrangente: fungos complexos, leveduras oxidativas, bactérias ácido acéticas, bactérias ácido láticas, enterobactérias, entre outras também fazem parte dessas fermentações, além das já conhecidas e tradicionais leveduras *Saccharomyces* e *Brettanomyces*. Os microrganismos identificados também foram relatados em diferentes estudos, como Bokulich et al. (2012), Spitaels et al. (2014), Bokulich et al. (2015), De Roos et al.(2019), De Roos et al. (2020), Shayevitz et al. (2020), Dysvik et al. (2020), Bossaert et al. (2021) e Tyakht et al. (2021), os quais também isolaram ou identificaram, através de sequenciamento de nova geração, o microbioma e microbiota de cervejas de fermentação mista.

A identificação através de sequenciamento de nova geração evita problemas encontrados em técnicas baseadas no isolamento dos microrganismos, uma vez que: (a) meios de cultivo utilizados podem ser seletivos a determinadas espécies, (b) microrganismos podem estar viáveis, contudo em um estágio não-cultivável e (c) espécies podem ter tempos de geração diferentes, fazendo com que diferentes tempos de incubação sejam necessários (Nocker et al. 2007; De Roos et al. 2020). Dessa forma, é possível identificar também microrganismos de difícil cultivo em laboratório, permitindo que toda comunidade microbiana presente na amostra em determinado período seja conhecida (De Roos et al. 2020).

A constante identificação de *Saccharomyces* spp. e *Brettanomyces* spp. em cervejas de fermentação mista deve-se a alguns fatores: ampla disponibilidade de *blends* comerciais baseados nessas leveduras; em fermentações espontâneas pode haver a contaminação cruzada com leveduras comerciais presentes no ambiente fabril; e dominância de leveduras desses gêneros frente a outras leveduras (Bokulich et al. 2012). No entanto, leveduras selvagens desses gêneros podem possuir maior avidez pelo consumo de substratos e/ou demonstrar características *killer*, produzindo compostos antimicrobianos e impactando negativamente o crescimento de leveduras comerciais (Canonico et al. 2014; Albergaria and Arneborg 2016; Rodhouse and Carbonero 2019). *Saccharomyces cerevisiae* reúne características que as permite dominar mostos de cerveja: alta capacidade fermentativa, crescimento em condições anaeróbicas e tolerância aos principais fatores de estresse, como alta osmolaridade, ácidos orgânicos e altas concentrações de etanol (Albergaria and Arneborg 2016; Molinet and Cubillos

2020). Assim como em nosso estudo, Tyakht et al. (2021) detectaram o mesmo padrão no microbioma de cervejas de fermentação mista: a predominância de *Saccharomyces* spp. e *Brettanomyces* spp., com outros gêneros com menor prevalência entre as amostras, como *Issatchenkia, Pichia, Hanseniaspora* e *Candida*).

Frequentemente, as leveduras não-*Saccharomyces* com maior predominância em cervejas de fermentação mista como Belgian Lambics e American Coolship Ales pertencem ao gênero *Brettanomyces*, principalmente da espécie *B. bruxellensis* (Bokulich et al. 2012; Spitaels et al. 2014; De Roos et al. 2019). A utilização de *Brettanomyces* spp. ganha destaque pois essas contribuem com aromas e flavors característicos (descritos como *funky*, tropicais, frutados, picantes), e se na presença de oxigênio, são capazes de produzir ácido acético em níveis facilmente detectáveis (Crauwels et al. 2015). No metagenoma das amostras avaliadas em nosso trabalho foi possível observar que em grande parte delas também foram detectadas leveduras desse gênero, com destaque para *B. bruxellensis*, a qual compunha quase a totalidade da amostra. Ainda, outras espécies como *B. custersianus* e *B. anomalus* foram encontradas em grandes proporções, as quais podem ser interessantes pois foi relatado que cepas dessas leveduras podem metabolizar celobiose e ter a atividade da enzima β-glicosidase (Colomer et al. 2020). Como demonstrado por De Roos et al. (2020), leveduras *Brettanomyces* têm grande participação em cervejas Lambic principalmente na fase de maturação, contribuindo com o típico *Brett flavor*, baseado na produção de compostos aromáticos como 4-etilguaiacol e 4-etilfenol.

Outras leveduras não-*Saccharomyces* podem ter papel fundamental na produção dessas cervejas. Assim como em nosso estudo onde observamos a grande presença de leveduras como *Wickherhamomhyces, Issatchenkia, Pichia* e *Lachancea* nas amostras, outros pesquisadores também detectaram dezenas de leveduras não-convencionais participando do processo fermentativo (Spitaels et al. 2014; Bokulich et al. 2015; De Roos et al. 2019; Tyakht et al. 2021). Essas leveduras, geralmente selvagens, estão presentes no ar e nas superfícies do ambiente fabril de produção da cerveja (Bokulich et al. 2012; Bokulich et al. 2015), no interior de barris utilizados durante a fermentação (De Roos et al. 2019), nas matérias-primas (Justé et al. 2011; Rodhouse and Carbonero 2019) ou então acabam sendo inoculadas a partir da adição de fontes como madeiras, especiarias, flores e frutas no mosto (Rodhouse and Carbonero 2019; Molinet and Cubillos 2020). Leveduras não-*Saccharomyces* podem apresentar um potencial inovador na indústria, como demonstrado por Osburn et al. (2017) que isolaram *W. anomalus* e *L. thermotolerans* a partir de amostras ambientais e as aplicaram de maneira controlada para fermentação de cerveja, identificando a capacidade de produzir cervejas de caráter ácido sem a necessidade da aplicação de bactérias ácido-láticas (técnica de *primary souring*).

5.2. A bioprospecção de leveduras e sua aplicação na fermentação de cervejas

A bioprospecção de leveduras para aplicação na indústria cervejeira busca diversos benefícios funcionais, como novos perfis de *flavors*, produção de bebidas com baixos níveis de

álcool, redução de calorias, acidificação por novos processos e o aprimoramento de novos estilos, resultando na diferenciação dos produtos (Cubillos et al. 2019). Ainda que seja uma opção atualmente discutida, a modificação genética de leveduras para aplicação na indústria cervejeira ainda não é permitida ou tem aplicação limitada em muitos países, o que destaca a importância da bioprospecção de novos isolados a partir da natureza (Alperstein et al. 2020). Centenas de espécies diferentes de leveduras podem ser isoladas em sua forma selvagem, desde aquelas encontradas em grande proporção em cervejas de fermentação mista quanto outras ainda não exploradas nessa aplicação. Em nosso trabalho foi possível obter diferentes isolados não-*Saccharomyces* a partir de amostras do Olympic National Park, dentre as quais *M. megachiliensis* demonstrou o maior potencial para aplicação na produção de cerveja após a caracterização fisiológica da levedura. Ainda, a partir de amostras como frutas e flores isolamos leveduras de gêneros comumente encontrados em fermentações espontâneas, como *Pichia, Hanseniaspora* e *Candida*.

A caracterização fisiológica dos isolados selvagens é de grande importância quando se objetiva sua aplicação em processos fermentativos. Como leveduras não-*Saccharomyces* são geralmente cepas que não passaram por um processo de domesticação, suas características fermentativas possuem grande variação das observadas para leveduras *Saccharomyces*, o que pode gerar incertezas e dificuldades em sua utilização (Capece et al. 2018). Características como a habilidade de utilizar diferentes açúcares do mosto e tolerância a condições de estresse são analisadas para predizer o comportamento das leveduras quando submetidas à fermentação de cerveja (Michel et al. 2016a).

Dentre as leveduras isoladas em nossos trabalhos, *M. megachiliensis* isolado ONP131 (ou *Trichosporoides megachiliensis*) foi caracterizada quanto diferentes aspectos fisiológicos, o que demonstrou sua capacidade de ser utilizada na fermentação de cervejas por tolerar as principais condições encontradas durante o processo. Essa é uma levedura pertencente ao filo Basidiomicota com reconhecida importância biotecnológica quando aplicada para produção de eritritol (Ghislain et al. 2002; Thoa et al. 2015), e a partir de nossos estudos, foi identificada pela primeira vez sua capacidade de fermentação de cervejas. Atualmente o mercado cervejeiro busca novas alternativas para produção de cervejas com baixo ou nenhum teor alcoólico, em que leveduras selvagens não-*Saccharomyces* são uma alternativa explorada (Cubillos et al. 2019). *Moniliella megachiliensis* é uma levedura que pode ser aplicada com essa finalidade, visto sua capacidade fermentativa reduzida quando comparado a *S. cerevisiae*, tolerância a altas concentrações de lúpulo e contribuição no aroma de cervejas. Sua utilização torna-se interessante pois algumas leveduras do gênero já foram caracterizadas quanto a atividade da enzima pectinase, importante principalmente na produção de cervejas com frutas (Priya and Sashi 2014; Singh and Kumar 2019). São previstos novos experimentos para avaliar a relação entre a produção de eritritol por *M. megachiliensis* e o aumento da sensação de boca (*mouthfeel*) em cervejas produzidas com a levedura. Ainda, novos estudos devem ser realizados buscando avaliar a segurança em sua utilização como cultura iniciadora para produção de bebidas fermentadas.

Leveduras não-convencionais representam um grupo de mais de 1500 espécies conhecidas (Gutiérrez et al. 2018). Várias dessas podem apresentar aplicação industrial, dependendo da sua caracterização fisiológica. Gêneros como *Hanseniaspora, Candida* e *Pichia* são constantemente identificados fazendo parte do microbioma de cervejas de fermentação mista (Spitaels et al. 2014; Spitaels et al. 2015; De Roos et al. 2020; Dysvik et al. 2020; Tyakht et al. 2021), o que demonstra sua habilidade em sobreviver nesse ambiente e participar do processo fermentativo, principalmente nas primeiras fases de fermentação onde há a ampla disponibilidade de oxigênio (Campbell 2003; Bokulich et al. 2012). Embora a contribuição dessas leveduras nos *flavors* durante fermentações mistas seja ainda pouco explorada, a partir do isolamento de cepas selvagens é possível caracterizá-las e identificar o perfil sensorial que atribuem em mostos de cerveja. Em nosso estudo foi possível observar que as três leveduras isoladas *Pichia kluyveri* LAR001*, Candida intermedia* ORQ001 e *Hanseniaspora uvarum* PIT001 têm baixa capacidade fermentativa, atenuando menos de 15% dos açúcares disponíveis no mosto. Ainda que não tenham atividade fermentativa como *S. cerevisiae* (atenuação superior a 70%), essas leveduras despertam o interesse por poderem ser utilizadas na produção de cervejas com baixo teor alcoólico (Saerens and Swiegers 2017) ou para o *bioflavoring,* isto é, potencializar os *flavors* através da utilização de microrganismos que produzem compostos voláteis em maiores concentrações (Holt et al. 2018).

Pichia kluyveri vêm sendo testada a partir de co-fermentações e fermentações sequenciais na produção de cervejas, sendo observado por Holt et al. (2018) e Ravasio et al. (2018) sua capacidade de aumentar aromas frutados que remetem a banana (Gutiérrez et al. 2018) a partir da conversão de compostos do lúpulo (Michel et al. 2016b). Esses aromas foram identificados em nosso trabalho, sugerindo que o isolado também pode ser aplicado para *bioflavoring*. Processos de produção de cervejas com baixo ou nenhum teor alcoólico utilizando isolados de *P. kluyveri* já foram patenteados (Saerens and Swiegers 2017) e ganham cada vez mais destaque (Iorizzo et al. 2021). Além da produção de cervejas, outras aplicações podem ser sugeridas para *P. kluyveri*, como na fermentação de cacau (Batista et al. 2015), tequila (Amaya-Delgado et al. 2013) e vinho (Lu et al. 2017). A aplicação dessa levedura em outros bioprocessos se beneficia da capacidade de crescimento de *P. kluyveri* em uma ampla faixa de pH, da tolerância a ácidos orgânicos e estresse iônico, e também de suportar temperaturas tão altas quanto 37 °C.

Hanseniaspora uvarum vem sendo identificada em fermentações mistas de cerveja em grandes proporções, como observado em cervejas Lambic belgas e *wild ales* no período de 1 a 4 semanas de fermentação (Spitaels et al. 2015; De Roos et al. 2020; Tyakht et al. 2021). O isolado PIT001 de *H. uvarum* foi responsável por aromas florais, frutados (remetendo a uva) e azedos/ácidos, os quais também foram identificados por Methner et al. (2019). Ainda que esses autores tenham considerado os aromas inaceitáveis ou desagradáveis, destacamos que os *flavors* produzidos pela levedura são de grande interesse em cervejas sour, American Coolship Ales e Belgian Lambics, portanto com grande potencial de aplicação em cervejas de fermentação mista. *H. uvarum* é geralmente isolada da superfície de uvas, em alguns casos compondo grande

parte da microbiota dessas frutas (Martin et al. 2018), e também de adegas de vinho (Grangeteau et al. 2016). Essa levedura é amplamente estudada quanto o *bioflavoring* exercido em vinhos (Martin et al. 2018), enquanto novos estudos abordam sua aplicação na produção de cervejas (Matraxia et al. 2021). Visto a susceptibilidade da levedura a condições adversas que podem ser encontradas em estágios tardios na produção de cerveja de fermentação mista (pHs muito ácidos e ácidos orgânicos), sugere-se seu uso nos primeiros dias de fermentação, quando há ampla disponibilidade de oxigênio e açúcares simples para consumo, e o pH do mosto está entre 4.0 – 6.0. Por outro lado, a capacidade de tolerar altas concentrações de ácidos-α do lúpulo permite que *H. uvarum* PIT001 seja utilizada em uma ampla gama de estilos de cerveja, como por exemplo India Pale Ales.

Leveduras pertencentes a *Candida* spp. podem não ser encontradas no microbioma de cervejas espontâneas, como observado em nosso trabalho, ou representando boa parte das leveduras identificadas em uma amostra, como observado Tyakht et al. (2021) e Spitaels et al. (2014). *Candida intermedia* já foi relatada contaminando cervejas (Pham et al. 2011; Hutzler et al. 2012), a qual é capaz de utilizar glicose, galactose, melezitose, sacarose, e algumas cepas ainda são capazes da fermentação de açúcares mais complexos, como maltose, trealose, celobiose e rafinose (Hutzler et al. 2012). Em nosso estudo foi sugerida pela primeira vez a utilização de *C. intermedia* de maneira controlada como levedura para fermentação de cervejas, visto a produção de aromas agradáveis e complexos, principalmente remetendo a madeira, especiarias, florais e fenólicos como cravo. Atualmente a principal aplicação pesquisada para *C. intermedia* está relacionada a sua grande capacidade de metabolização de xilose (açúcar lignocelulósico), monossacarídeo que *S. cerevisiae* é incapaz de fermentar naturalmente (Geijer et al. 2020). Essa atividade é interessante pois pode implicar em novos *flavors* sendo gerados a partir da incubação de cervejas fermentadas com *C. intermedia* em barris de madeira. Leveduras halotolerantes têm o controle sobre os efeitos combinados de baixa atividade de água, pressão osmótica e alto estresse iônico (Silva-Graça and Lucas 2003). No caso de *C. intermedia*, essa característica aliada a tolerância a presença de ácido lático favorecem seu uso em cervejas de estilos tradicionais (ex: Gose) que necessitam da adição de grande quantidade de sais e pH ácidos em torno de 3.5, ou então na produção de vegetais fermentados (Bonatsou et al. 2015). Essa levedura pode ainda ser utilizada em co-fermentações visando manter culturas restritas a determinadas associações de microrganismos, como observado por Peña et al. (2020) os quais demonstraram que *B. bruxellensis* e *Pichia guilliermondii* são sensíveis a peptídeos antimicrobianos produzidos por cepas de *C. intermedia* enquanto o crescimento de *S. cerevisiae* não sofre impacto.

5.3. Outras aplicações: potencial probiótico de leveduras não-*Saccharomyces*

Leveduras são comercializadas há décadas como suplementos alimentares por causa do seu alto conteúdo de vitamina B, proteínas, peptídeos e aminoácidos (Foligné et al. 2010). Além disso, a atividade antimicrobiana apresentada por leveduras pode ser o indício de outros

atributos que o microrganismo possui. Dentre eles, o potencial para atividade probiótica. Leveduras probióticas podem ser responsáveis por benefícios à saúde do hospedeiro, ao passo que podem modular o sistema imune para sua homeostase, influenciar na imunidade adaptativa, ou manter o equilíbrio da microbiota do intestino por meio de interações específicas (Sen and Mansell 2020). *Saccharomyces boulardii* é uma levedura com atividade probiótica de eficácia comprovada, sendo utilizada em diversos países como agente de prevenção e terapêutico contra diarreia e outras desordens no trato gastrointestinal causadas pela administração de antimicrobianos (Czerucka et al. 2007; Sen and Mansell 2020). Foram propostos diferentes mecanismos de ação para o efeito probiótico dessa levedura, entre eles: potencialização da imunidade inata, modulação do sistema imune através da regulação da secreção de citocinas, estímulo do aumento de junções de oclusão da barreira epitelial e competição com microrganismos patogênicos por sítios de adesão na mucosa intestinal (Lukaszewicz 2012; Sen and Mansell 2020). Assim como essa, outras leveduras podem ter potencial de aplicação como microrganismo probiótico, entre elas leveduras não-*Saccharomyces* (Fernández-Pacheco et al. 2021a; Staniszewski and Kordowska-Wiater 2021).

Em nosso estudo observamos a capacidade de *P. kluyveri* LAR001, *H. uvarum* PIT001 e *C. intermedia* ORQ001 em inibir o crescimento de patógenos bacterianos relacionados aos alimentos, bem como em agregarem-se a esses patógenos. A habilidade de competir por nutrientes (Gross et al. 2018), inibir o crescimento de outros microrganismos (Labbani et al. 2015; Cordero-Bueso et al. 2017), co-agregação (Ogunremi et al. 2015b) e resistir as condições do TGI (Bonatsou et al. 2015) já foi observada para outras cepas de *P. kluyveri*, sendo sugerida sua utilização para biocontrole e potencial probiótico. *Hanseniaspora uvarum* tem destaque principalmente devido sua aplicação e identificação em alimentos e bebidas fermentadas (Lara-Hidalgo et al. 2017), contudo cepas costumam ser descartadas durante a seleção de leveduras para caracterização do potencial probiótico (Goerges et al. 2006; Ogunremi et al. 2015b; Fernandez-Pacheco Rodríguez et al. 2018b). Fernandez-Pacheco Rodríguez et al. (2019) caracterizaram o potencial probiótico de espécies de *Hanseniaspora,* enquanto Agarbati et al. (2020) e Ma et al. (2013) demonstraram a capacidade de inibirem o crescimento de patógenos, como *C. albicans, S. aureus, Shewanella marisflavi, Vibrio splendidus*. Ainda que Cassanego et al. (2017) não tenha identificado a resistência de isolados de *H. uvarum* a simulação da digestão pancreática, essa habilidade foi identificada no isolado PIT001 em nosso trabalho. Foi observado por Agarbati et al. (2020) a incapacidade de *H. uvarum* tolerar a temperatura de 37 °C para crescimento, o que também foi identificado para *H. uvarum* PIT001. Diferentes cepas da levedura *C. intermedia* vem sendo relatadas quanto a alta capacidade de inibição de patógenos, como demonstrado por Goerges et al. (2006) na inibição de *L. monocytogenes* e por Younis et al. (2017) na atividade antagonista a *E. coli*, *S. aureus* e *P. aeruginosa*. Nossos resultados corroboram com esses dados, visto que *C. intermedia* ORQ001 foi capaz de inibir o crescimento de diferentes patógenos e apresentar bons níveis de co-agregação (≥ 66%) com os mesmos.

No caso de *S. boulardii*, a atividade imunomodulatória exercida pelo microrganismo e seus derivados também desperta o interesse em seu uso (Jensen et al. 2008; Tewary and Patra

2011), principalmente por direcionar o sistema imune a uma resposta anti-inflamatória durante infecções (Foligné et al. 2010; Stier and Bischoff 2016). Pouco se sabe sobre milhares de leveduras não-*Saccharomyces* quanto sua interação com o sistema imune mucosa baseado no trato gastrointestinal (Smith et al. 2014). Nosso trabalho buscou explorar como células do sistema imune reagem a *H. uvarum, P. kluyveri*, *C. intermedia* e seus derivados, visto que o reconhecimento e resposta por essas células pode ser variável dependendo do estímulo. Ainda que grande parte dos efeitos benéficos das leveduras sejam atribuídos a componentes estruturais da parede celular (β-glicanos e mananoligossacarídeos, por exemplo), metabólitos ativamente expressos por essas células (aminoácidos, peptídeos, vitaminas) também têm sido relacionados a atividade probiótica desses microrganismos (Ran et al. 2015).

Nosso trabalho foi o resultado de um dos primeiros estudos referente ao reconhecimento das leveduras *P. kluyveri*, *H. uvarum* e *C. intermedia* por células imunes. Após o estímulo de macrófagos RAW 264.7 observamos que células vivas de *C. intermedia* são responsáveis por induzir uma transcrição relativa de mRNA significante das citocinas IL4 e IL13, assim como *S. boulardii*, que além dessas citocinas foi capaz de induzir a expressão de IL2 e IL23 em níveis superiores ao observado para as demais leveduras. Embora a resposta imune a diferentes leveduras do gênero *Candida* seja amplamente estudada, se observa que o reconhecimento é espécie-específico (Smith et al. 2014; Bazan et al. 2018), dessa forma torna-se difícil predizer o padrão de resposta imune baseado em outras espécies do mesmo gênero. Por exemplo, como observado por Saegusa et al. (2007) no estímulo de células epiteliais intestinais *in vitro*, os níveis de expressão de IL8 são detectados em concentração variável entre leveduras patogênicas e não-patogênicas, com destaque para as variações observadas quando espécies de *Candida* foram utilizadas.

Células vivas de *P. kluyveri* e *H. uvarum* foram responsáveis pela indução da expressão de fatores de transcrição como Bcl6 e STAT3, embora tenham induzido somente baixos níveis de expressão das citocinas estudadas. Bcl6 é fundamental na regulação de uma resposta Th2, reprimindo a expressão da citocina pró-inflamatória IL6 (Toney et al. 2000; Li et al. 2020). STAT3 participa da mediação de efeitos anti-inflamatórias de IL10, também restringindo a expressão de citocinas pró-inflamatórias (Lang 2005; Hillmer et al. 2016). A indução da transcrição de mRNA desses fatores também foi induzida por *C. intermedia* e *S. boulardii*, contudo em menor intensidade quando comparado às leveduras citadas anteriormente. O aumento da expressão de Bcl6 durante o estímulo de células imunes com leveduras foi identificado também por Santos et al. (2021), corroborando com os dados encontrados em nosso trabalho.

Diversas estruturas presentes na parede celular de leveduras podem estimular a expressão do receptor TLR2 em fagócitos e células dendríticas (Romani 2004). Baseado nas diferenças de composição e concentração das moléculas da parede celular entre as espécies de leveduras (Lozančić et al. 2021) foi sugerido que o reconhecimento por macrófagos pode ocorrer de maneira distinta. Nesse sentido, investigamos a transcrição relativa de mRNA do receptor TLR2, a partir do qual identificamos que o estímulo com células vivas de *P. kluyveri* foi

responsável por altos níveis de expressão do receptor em macrófagos. Outros receptores, como Dectin-1, são responsáveis pelo reconhecimento de fungos (Figueiredo et al. 2011), dessa forma mais estudos serão conduzidos para identificar a expressão desses receptores após o estímulo com células vivas das leveduras.

Células inativadas das leveduras também foram utilizadas para o estímulo dos macrófagos. Buscamos dessa forma avaliar a resposta mediada em células imunes sem que compostos metabolicamente ativos possam ser secretados pelas leveduras durante o período de estímulo. Células inativadas também podem ter efeitos imunomodulatórios, como já demonstrado para bactérias e leveduras de potencial probiótico (Ou et al. 2011; Smith et al. 2014). O tratamento de inativação por calor é responsável pela exposição de moléculas que podem ser imunologicamente ativas, como quitina e β1,3-glicanos (Navarro-Arias et al. 2019), sendo possível observar alterações na produção de citocinas por células imunes durante o reconhecimento de células vivas ou inativadas (Navarro-Arias et al. 2019). Em nosso estudo observamos que os níveis das citocinas IL2, IL4, IL13, e IL23 mantêm-se basais quando células inativadas de *S. boulardii* e *C. intermedia* foram utilizadas como estímulo, diferindo do detectado para células vivas dessas leveduras. Após o tratamento com calor, células de *H. uvarum* foram capazes de estimular níveis significantes de IL10 e IL23, *P. kluyveri* estimulou níveis maiores de IL4 e *C. intermedia* da citocina TNF-α. A transcrição relativa de mRNA de fatores de transcrição foi observada em níveis superiores quando células inativadas foram utilizadas, o que também evidencia que modificações estruturais resultantes do tratamento com calor podem influenciar no reconhecimento por células imunes e em cascatas de sinalização. As diferenças observadas nas respostas podem indicar o possível efeito paraprobiótico dessas cepas, o que deve ser investigado em experimentos futuros. Assim como observado em células vivas, células inativadas de *P. kluyveri* e *H. uvarum* também foram responsáveis por estimular níveis significantes de TLR2, demonstrando que o estímulo do receptor em macrófagos manteve-se similar ainda que o tratamento com calor possa causar alterações na estrutura da célula.

Metabólitos secretados por leveduras também são relacionados ao efeito imunomodulatório desempenhado por esses microrganismos (Ran et al. 2015; Marcos et al. 2016), recebendo a denominação de pós-bióticos (Nataraj et al. 2020). Peptídeos secretados por *C. albicans* são responsáveis pela ativação de respostas inflamatórias baseadas no reconhecimento via TLR2/TLR4 durante a invasão do hospedeiro (Wang et al. 2019), o que também já foi evidenciado para outros fungos patogênicos (Marcos et al. 2016). Produtos do secretoma dessas leveduras estão relacionados a regulação do sistema imune do hospedeiro, sendo importantes fatores de virulência (Rasheed et al. 2020). Dessa forma, o sobrenadante dos cultivos de *H. uvarum*, *P. kluyveri* e *C. intermedia* também foram avaliados quanto seu efeito imunoestimulatório. Em nossos experimentos os sobrenadantes não foram capazes de estimular níveis significativos de transcrição relativa de mRNA das citocinas e dos fatores de transcrição avaliados, contudo foi observado que a transcrição do mRNA de TLR2 foi detectada em níveis significativos quando o sobrenadante de *C. intermedia* foi utilizado como estímulo, sugerindo a presença de peptídeos que estimulam o reconhecimento pelo receptor. Peptídeos

antimicrobianos e toxinas *killer* presentes no secretoma de *H. uvarum* (Mehlomakulu et al. 2015), *P. kluyveri* (Vicente et al. 2021) e *C. intermedia* (Peña et al. 2020) despertam o interesse de pesquisadores que investigam o potencial dessas leveduras para biocontrole na produção de bebidas, porém estudos voltados para a interação de moléculas do secretoma com células do sistema imune ainda não escassos.

Em nosso trabalho buscamos investigar quais derivados das leveduras tinham potencial imunoestimulatório, entre eles o DNA. O reconhecimento de RNAs de dupla fita, ssRNA e DNA CpG hipometilado ocorre geralmente por TLRs (TLR3, TLR7, TLR8 e TLR9), induzindo a expressão de genes pró-inflamatórios como interferons do tipo I (Atianand and Fitzgerald 2013). DNAs CpG de fungos fazem parte dos MAMPs que podem ser encontrados em microrganismos probióticos, responsáveis por estimular principalmente TLR9 (Pathakumari et al. 2020) e que participam da imuno-ativação e imunossupressão do sistema imune (D. Foey 2018). A partir do estímulo de macrófagos com DNAs purificados das leveduras observamos que as transcrições relativas de mRNA de citocinas, fatores de transcrição, *YM1* e *TLR2* foram próximas de níveis basais, com exceção de *IL10*, a qual demonstrou baixos (porém significantes) níveis de transcrição a partir desses estímulos. Roberts et al. (2005) demonstraram que o DNA genômico de bactérias pode ser captado com maior facilidade que DNAs CpG por macrófagos. A indução da expressão de IL10 foi observada também por Lammers et al. (2003) em PBMCs estimulados com DNA genômico de bactérias probióticas, como *Lactobacillus, Bifidobacterium* e *Streptococcus*. Novos estudos devem ser realizados quanto a transcrição de outros receptores específicos (ex: TLR9), bem como diferentes concentrações de DNA, visto que a secreção de citocinas pode variar de acordo com a concentração de DNA utilizada para estímulo das células imunes (Lammers et al. 2003).

Para sua aplicação como leveduras probióticas, novos estudos devem ser conduzidos referentes a segurança em sua aplicação, como o desenvolvido por Fernández-Pacheco et al. (2021b) com diferentes espécies de *Pichia, Saccharomyces, Hanseniaspora, Candida, Lachancea* e *Zygosaccharomyces* observando a produção de aminas biogênicas, resistência a antifúngicos, atividade enzimática, entre outros testes. Assim, os resultados obtidos nesse trabalho revelam de forma preliminar o potencial probiótico dos isolados *P. kluyveri* LAR001, *C. intermedia* ORQ001 e *H. uvarum* PIT001, enquanto novos estudos devem ser realizados *in vivo* quanto seu potencial imunoestimulatório e de combate a patógenos.

6. **Conclusão**

A bioprospecção de microrganismos a partir do ambiente e de fermentações espontâneas pode revelar microrganismos ainda pouco explorados para aplicação em processos fermentativos, havendo a necessidade de isolá-los e caracterizá-los para conhecer seus atributos e características. Leveduras selvagens não-*Saccharomyces* despertam o interesse em seu estudo pois podem conferir novos *flavors* às cervejas, ser tolerantes a diferentes condições de

estresse que beneficiam sua aplicação em processos fermentativos específicos, e ainda podem possuir potencial probiótico, entre muitas atividades agem combatendo patógenos e estimulando o sistema imune. Nesse trabalho foi possível isolar e caracterizar fisiologicamente as leveduras *Moniliella megachiliensis* (ONP131)*, Pichia kluyveri* (LAR001)*, Hanseniaspora uvarum* (PIT001) e *Candida intermedia* (ORQ001), apresentando potencial de aplicação na produção de cervejas. Além disso, os isolados *P. kluyveri, H. uvarum* e *C. intermedia* apresentam potencial probiótico, principalmente, quanto a atividade antimicrobiana e ação imunoestimulatória em macrófagos *in vitro*.

Identificamos que todas as leveduras demonstraram potencial para fermentação de cervejas, sugerindo a utilização de maneira isolada ou a partir de co-fermentações. Foi observado nessas leveduras também o potencial probiótico e paraprobiótico, pois foram capazes de inibir o crescimento de patógenos bacterianos, resistiram às condições adversas do trato gastrointestinal e foram consideradas imunologicamente ativas pelo reconhecimento por células do sistema imune. Dessa forma concluímos que leveduras não-convencionais podem representar alternativas interessantes para o desenvolvimento de bebidas inovadoras, além de também possuírem potencial de aplicação como microrganismos probióticos. Como perspectivas futuras, objetivamos utilizar as leveduras isoladas para produção de cervejas em maior escala, caracterizando os compostos voláteis produzidos. Ainda, testes *in vivo* em modelos animais deverão ser realizados para confirmar a atividade probiótica das leveduras.

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