TATIANE DE PAULA SIQUEIRA

ISOLATION AND CHARACTERIZATION OF YEASTS FROM BRAZILIAN COFFEE BEANS FOR BREWING APPLICATION

Thesis presented to Federal University of Viçosa as part of the requirements of the Postgraduate Program in Agricultural Microbiology, to obtain the title of *Doctor Scientiae*.

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BIOGRAPHY

TATIANE DE PAULA SIQUEIRA, Sílvia Perpétua de Siqueira's daughter, was born in Bom Sucesso, Minas Gerais, on June 2, 1995. Her studies at the Federal University of Viçosa have been started on March 2014. She obtained the grade in Biological Sciences in July 2017. One month later, she began her master's study in the Agricultural Microbiology Graduate Program, obtaining the Master grade in December 2018. Since January 2019, she has been developing the Doctorate study at the same Program.

SUMMARY

ABSTRACT

SIQUEIRA, Tatiane de Paula, M.Sc./D.Sc., Universidade Federal de Viçosa, November, 2023. **ISOLATION AND CHARACTERIZATION OF YEASTS FROM BRAZILIAN COFFEE BEANS FOR BREWING APPLICATION**.

The use of new non-*Saccharomyces* yeasts is a strategy for obtaining beers with new sensory profiles. This makes it possible to attend to the expectations of consumers who are increasingly looking for innovative beverages. In this study, we proposed the isolation of yeasts from coffee fruits, samples that have a wide range of microbial diversity. The study was conducted on samples obtained from the Alto da Mogiana region, a place in Minas Gerais known for its high-quality coffees. Twenty-seven isolates were obtained and of these, 52 % were able to use maltose as a carbon source and 87.5 % exhibited low hydrogen sulphide production. In addition, most of the isolates exhibited tolerance to factors such as alcohol content, low pH and temperature variation. Based on these results, two isolates (F702 and F605) were identified and selected for laboratory-scale fermentation characterization. Isolate F605 belongs to the *Wickerhamomyces anomalus* species, while F702 is a strain of *Torulaspora delbrueckii*. Laboratory fermentation trials have shown that these yeasts are unable to attenuate beer wort and, consequently, do not produce ethanol. In this context, the use of isolate F605 in co-fermentation with a conventional yeast was proposed. The F605 isolate was selected because of the better sensory profile observed throughout the experiments. The beer produced had an alcohol content of 5.6 %, indicating that the conventional yeast was able to grow and ferment in the presence of the isolate. An assay was also carried out to check consumer acceptance, which showed positive evaluations for criteria such as appearance and aroma. It can therefore be concluded that the coffee fruits evaluated were promising environments for isolating yeast with potential for application in the brewing industry. This reinforces the importance of sustainably exploiting Brazilian environments to obtain new national brewing strains, which can generate financial returns for the economy, as well as scientific and technological advances.

RESUMO

SIQUEIRA, Tatiane de Paula, M.Sc./D.Sc., Universidade Federal de Viçosa, novembro, 2023. **ISOLAMENTO E CARACTERIZAÇÃO DE LEVEDURAS OBTIDAS DE GRÃOS DE CAFÉS BRASILEIROS PARA APLICAÇÃO NA PRODUÇÃO DE CERVEJAS.**

A utilização de novas leveduras não-*Saccharomyces* é uma estratégia para obtenção de cervejas com novos perfis sensoriais. Isso permite atender às expectativas dos consumidores que buscam cada vez mais bebidas inovadoras. Neste estudo, foi proposto o isolamento de leveduras de frutos de café, amostras que apresentam ampla diversidade microbiana. O estudo foi realizado em amostras obtidas na região do Alto da Mogiana, local de Minas Gerais conhecido por seus cafés de alta qualidade. Foram obtidos 27 isolados e destes, 52 % foram capazes de utilizar maltose como fonte de carbono e 87,5 % apresentaram baixa produção de sulfeto de hidrogênio. Além disso, a maioria dos isolados apresentou tolerância a fatores como teor alcoólico, baixo pH e variação de temperatura. Com base nestes resultados, dois isolados (F702 e F605) foram identificados e selecionados para caracterização fermentativa em escala laboratorial. O isolado F605 pertence à espécie *Wickerhamomyces anomalus*, enquanto F702 é uma cepa de *Torulaspora delbrueckii*. Os ensaios de fermentação em laboratório demostraram que essas leveduras são incapazes de atenuar o mosto da cerveja e, consequentemente, não produzem etanol. Neste contexto, foi proposta a utilização do isolado F605 em co-fermentação com levedura convencional. O isolado F605 foi selecionado devido ao melhor perfil sensorial observado ao longo dos experimentos. A cerveja produzida apresentou teor alcoólico de 5,6 %, indicando que a levedura convencional foi capaz de crescer e fermentar na presença do isolado. Também foi realizado um ensaio para verificar a aceitação do consumidor, que apresentou avaliações positivas para critérios como aparência e aroma. Pode-se concluir, portanto, que os frutos de café avaliados foram ambientes promissores para isolamento de leveduras com potencial para aplicação na indústria cervejeira. Isso reforça a importância da exploração sustentável dos ambientes brasileiros para a obtenção de novas linhagens cervejeiras nacionais, o que pode resultar em retornos financeiros para a economia, bem como avanços científicos e tecnológicos.

1. INTRODUCTION

Beer is one of the most consumed alcoholic beverages in the world and its production process is considered one of the oldest and most important biotechnological events of humanity. The relevance of beer production is related to its role on the first agricultural practices (Liu et al., 2018; Wannenmacher et al., 2018), been reported since since 5500 B.C. in both Egypt and Mesopotamia (Callejo et al., 2017). Furthermore, recent studies have suggested Israel as the origin place where beers were spread for other countries. Liu et al. (2018) found traces of alcohol in caverns dating back more than 13,000 years, making them the oldest accounts of beer production.

Although these beverages have been created thousand years ago, it was only in 1861 that Louis Pasteur elucidated the importance of microbial metabolism in the fermentative process (Karabín et al., 2017). This discovery allowed the development of new techniques applied for brewing, such as the use of starter cultures by Emil Christian Hansen in 1883 (Petruzzi et al., 2016; Karabín et al., 2017).

In this context, beer has gained prominence; nowadays, it consists of the third most popular beverage in the world, behind only coffee and tea (Capece et al., 2018). In Brazil, the traditional brewing industry has presented high production, occupying the third position in the world ranking of largest beer producers, with 13.3 billion liters/year, behind only China and the United States (Sindcerv, 2020).

For beer production, barley malt, water, *Humulus lupulus* flowers and yeast are used as ingredients. The main yeast strains employed in the production of Ale and Lager beers consists of *Saccharomyces cerevisiae* and *Saccharomyces pastorianus*, respectively (Bamforth, 2017). These microorganisms convert fermentable sugars available in the wort into alcohol, CO_2, and several products. Both species are considered as conventional yeasts, being widely used due to their profile compounds profile, as well as the fast metabolism, efficiency in alcohol production and tolerance to stress conditions (Basso et al., 2016; Gallone et al., 2016).

However, considering the increasing demand for beverages with more complex sensory profiles in last few years, the use of non-*Saccharomyces* yeasts became a trend in the brewing industry (Basso et al., 2016). Among them,

Brettanomyces, *Wickerhanomyces*, *Torulaspora*, *Kluyveromyces* and *Hanseniaspora* have been suggested for use in brewing processes (Basso et al., 2016; Domizio et al., 2016; Gibson et al., 2017). Therefore, non-*Saccharomyces* yeasts can contribute to the production of innovative beers, functional or low-alcohol beverages (Basso et al., 2016). In addition to be the main fermenting microorganism, non-*Saccharomyces* yeasts can also be used as co-fermenters; this practice has been used in wine production and suggested for brewing proposals (Gibson et al., 2017).

Therefore, the identification of new yeasts with potential for use in industry is a field of interest for various research groups. This prospecting can take place through different processes, such as the natural fermentation of fruits and grains. Coffee beans can be fermented for improving its sensory quality. In general, coffee fermentation is provided by controlling autochthonous microorganisms in the beans, although starter cultures can also be used (Humia et al., 2019). Several microbial groups can play important roles on coffee fermentation, but yeasts are the most important (Pereira et al, 2017), including *Pichia*, as well as *Saccharomyces*, *Torulaspora*, *Kloeckera* and *Hanseniaspora* (Humia et al., 2019).

The fermentation process results in the improvement of compound content related to sensory characteristics of interest, such as organic acids, higher alcohols, aldehydes and esters (Lee et al., 2015; Pereira et al, 2017). Thus, the prospection of yeasts from coffee beans has been stimulated in order to elaborate beverages with more complex sensorial profiles (Haile & Hang, 2019).

The characterization of novel strains isolated from coffee beans can bring interesting insights about their role on beverage quality, allowing their use in the production of other fermented beverages, such as innovative beers.

2. REVIEW: What do yeasts need to produce beer? An updated review

The brewing process is one of the most important and oldest biotechnological practices, playing a relevant role for the development of the agriculture (Liu et al., 2018; Wannenmacher et al., 2018). Currently, beer is among the most consumed alcoholic beverages in the world (Ravasio et al., 2018), and it is the third most popular, behind only tea and coffee (Capece et al., 2018). In 2022, 1.89 billion hectoliters of beer were produced worldwide (Statista, 2022). China, United States and Brazil are the leading countries in the ranking of largest producers (Statista, 2022). The global beer market is expected to exceed US$ 600 billion by 2025 (Allied Market Research, 2018).

For beer production, water, barley malt, hops, and yeast are used as ingredients (Olajire et al., 2012). Several types of adjuncts may also be applied, according to the standards established by the local roles (Cela et al., 2020). Adjuncts include wheat, wheat malt, corn, rice, as well as fruits and spices (Alves et al., 2020; Calvo-Porral et al., 2020; Nardini et al, 2020). Regarding microorganisms, *Saccharomyces*-species are the most common due to their efficiency in converting sugars into ethanol, producing flavors of interest for the final product (Gallone et al., 2016).

Beer production begins with the malting process, in which the maturation of the enzymes required for the conversion of starch into simple sugars is observed (Alves et al., 2020). Subsequently, the malted grains are milled to obtain a high yield of extracted substances, a process called milling. Then, the mashing step is performed by heating the grains in water through infusion or decoction, resulting in the wort (Olajire, 2020).

Afterward, the wort is boiled, followed by the hopping process, in which *Humulus lupulus* flowers are added to provide aroma and bitterness to the beverage (Pascari et al., 2017). Hops also present antimicrobial activity, playing an important role in controlling undesirable bacteria during the brewing process (Steenackers et al., 2015). Then, coagulated proteins and hop residues are eliminated from the wort with the trub separation, followed by cooling and aeration steps (Alves et al., 2020). Finally, yeasts are inoculated to start the fermentation process (Figure 1). These microorganisms are responsible for the conversion of the sugars into ethanol and

carbon dioxide, as well as into several sensory compounds (Alves et al., 2020; Kerr et al., 2018).

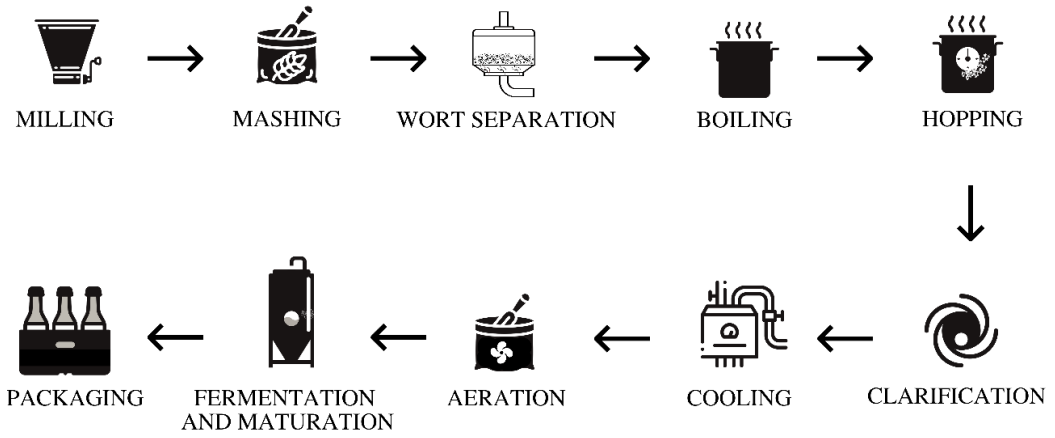


Figure 1. Summary scheme of the steps involved in beer production: Beer is produced from malted barley, which undergoes a milling process to expose the starch in the grains. These grains are then mixed with hot water in a process known as mashing, where enzymes convert starch into fermentable sugars. The resulting liquid, known as wort, is boiled, adding hops to provide characteristic bitterness and aroma. After cooling, the wort is transferred to fermentation, where yeasts are added to convert the sugars into alcohol and carbon dioxide. The resulting liquid, now called green beer, is matured to enhance the flavor. The final stage involves bottling or kegging.

After the fermentation, yeast is removed from the fermentation tank; in some cases, a secondary fermentation process is carried out, in which more sugar and yeast can be added, promoting a stronger carbonation. Finally, the beverage is submitted to a maturation step at low temperature; it's important to promote the sedimentation of the yeast and other compounds, resulting in a clarified beer (Olajire, 2020).

The diversity of raw material, as well as the processes involved in the brewing production result in a range of beverages with a wide diversity of sensory compounds. The composition of ingredients, type of malt, mash step conditions, yeast strains and pitching rates, aeration level, fermentation, maturation and storage conditions can impact the beverage characteristics (Kucharczyk et al., 2016; Alves

et al., 2020). Therefore, it is possible to produce a wide diversity of beers, which differ in sensory profile, color, turbidity, foam, and other characteristics of interest (Bamforth, 2017; Alves et al., 2020; Pieczonka et al., 2020).

The strain used for beer production plays an important role in the beer quality. They must be adapted to the conditions established in the production process, since they are able to ferment quickly and without producing off-flavors. In this context, the aim of this topic is present an updated review about the main properties of the strains involved in the brewing industry and its impacts on the beer quality.

2.1. Brewer's Yeast

The role of microbial fermentation for beer production was elucidated in 1861 by the Louis Pasteur studies (Pasteur, 1876). Until that moment, the wort was spontaneously fermented by a complex mixture of autochthonous species (Karabín et al., 2017). As science advanced, pure cultures were introduced in the brewing practice, especially *S. cerevisiae* and *S. pastorianus* strains (Bamforth, 2017). These species are the most common due to their sensory profile, as well as the quick use of sugar from the wort (Gallone et al., 2016).

Saccharomyces is a yeast widely used in biotechnological processes. These yeasts were exposed to centuries of a domestication process in which wild type strains were transformed over time into a more adapted strains that perform better activities of interest (Gallone et al., 2018; Fay et al., 2019).

Gallone et al. (2016) evaluated the profile of microorganisms commonly employed in industrial processes through DNA sequencing. They showed that yeasts can be evolutionarily separated into five clades, composed of: i) Asian strain; ii) wine-producing yeast; iii) mixed clades with bread and others yeasts; iv) and v) and two clades composed by brewing strains. Clear evidence of microbial domestication can be highlighted, such as polyploidy pattern, gene duplication and chromosome rearrangements (Driscoll et al., 2009; Purugganan and Fuller, 2009). The clades of brewer's yeasts were strongly influenced by geographical parameters, resulting in distinct changes from the others (Gallone et al, 2016). Besides that, the domestication was considered most pronounced in brewer’s yeasts, mainly due to their constant use for food and beverage production, preventing the contact with

natural environments. During the time, this process reflected in genome decay, aneuploidy, and loss of the sexual cycle (Gallone et al., 2016). Moreover, several modifications were detected in the yeast genome, such as duplication, deletion, and gene recombination. These changes are mainly present in the telomeric region of the chromosomes, which is a strong indication of evolution (Brown et al., 2010; Gallone et al., 2016). As the result, these microorganisms present higher fermentative performance, ethanol production and tolerance, better osmotic stress tolerance, absence of phenolic profile, appropriate flocculant profile and preference for fermenting in the presence of oxygen (Mukai et al., 2014; Fay et al., 2019; Gibson et al., 2020).

S. cerevisiae is the most industrially exploited microorganism and the main one used in the production of ale-type beers (Bamforth et al., 2017; Menezes e Silva, 2019). It presents relevant properties for beer production such as adaptation to survive in sugar-rich environments and with low oxygen concentrations, and the ability to metabolize maltotriose abundant in beer wort – a great evidence of the domestication process, since it is not abundant in natural environments (Kato & Takahasi, 2022).

In turn, *S. pastorianus*, a hybrid microorganism resulting from the union of *S. cerevisiae* and *S. eubayanus*, is used for lager beer production (Libkind et al. 2011). Their hybridization is considered a spontaneously phenomenon in brewing environments, where a *S. cerevisiae* strain used in beer production combined with a *S. eubayanus* contaminant, resulting in a strain able to ferment the wort at low temperatures (Gorter de Vries et al., 2021).

Strains used to ferment lager beers are generally employed at low temperatures (6 to 15 °C), while ale strains perform better at higher temperatures (16 to 24 °C) (Lasanta et al., 2020). Differences can be observed for their flocculant profiles. Flocculation is related to the cell aggregation, resulting in flocs that no longer remain in suspension, indicating the end of beer fermentation (Speers, 2016). This property is very important in beer production process, as it facilitates the yeast separation from the wort, leaving the beer brighter and with reduced microbial load (Kerr et al., 2018; Kayacan et al, 2020). *S. cerevisiae* flocculates at the top of the fermentation tank; on the other hand, lager strains generate flocs which remain at the bottom (Kerr et al., 2018).

Brewer's yeasts are also characterized by using quickly several carbon and energy sources from the wort, such as glucose, sucrose, fructose, maltose, galactose, raffinose, maltotriose and realose (Marongiu et al., 2015). Besides that, yeasts used for lager beer production also use exclusively melibiose (Olaniran et al., 2017). In general, the first sugars consumed are sucrose, glucose and fructose, followed by maltose, and finally maltotriose (Brickwedde, 2019). In this context, the availability of them influences directly the microbial metabolism and, consequently, the beverage quality. Other factors such as genetic properties of the strain, pitching rate, and fermentation conditions also play an important role for brewing (Stewart et al., 2018).

2.2. Brewer's Yeast Properties

2.2.1. Sugar Assimilation

The grains used in beer production are rich in carbohydrates, predominantly as starch. During the first steps of brewing process, they are hydrolyzed into simple sugars resulted by amylase activity (Brickwedde, 2019). Then, maltose and maltotriose are the main compounds generated, corresponding to about 60 % and 20 % of the total sugars in the wort, respectively. The others compounds are represented by dextrins, glucose, fructose and sucrose (He et al., 2014; Brickwedde, 2019).

The main role of brewer's yeast is to convert sugars into ethanol and carbon dioxide, quickly and without off-flavors generation (James et al., 2003). After sucrose, monosaccharides such as glucose and fructose are assimilated via hexose transporters, encoded by genes such as Hxt1 and Hxt17. Glucose molecules, for example, are transported without energy expenditure by facilitated diffusion (Hatanaka et al., 2018). After depleting them, disaccharides and trisaccharides such as maltose and maltotriose are consumed (He et al., 2014). Their transportation into the yeast cell occurs by symport system, which depends on the electrochemical proton gradient, consuming energy (Leeuwen et al., 1992; Cousseau et al., 2013).

The assimilation of α-glucosides, such as maltose, depends on the expression of genes from MAL *locus*, which encodes for α-glucoside transporters, α-glucosidase and transcriptional activation proteins (Needleman et al., 1984;

Charron et al., 2001). They are present in more copies in the genome of brewer's yeasts, representing clear evidence of adaptation for industrial purposes (Gallone et al., 2016). For *S. cerevisiae,* six different maltose transporters, encoded by *MAL21, MAL31, MAL61, AGT1, MPH2* and *MPH3* genes, are known. Among them, three genes (*AGT1, MPH2* and *MPH3*) are less specific, being capable of transporting maltotriose (Day et al., 2002). Inside the cell, both maltose and maltotriose can be hydrolyzed by maltases (Cousseau et al., 2013).

Maltotriose uptaking occurs slower than maltose due to differences on affinity for transportation systems (Stambuk et al., 2001). Thus, maltose can act as an inhibitor of maltotriose transport (Day et al., 2002). It is noteworthy that when maltotriose is not fully assimilated, sensory defects such as excessive sweetness can be observed, in addition to ethanol yield decreasing (Salema-Oom et al., 2005). Therefore, the evaluation of maltotriose absorption and utilization is a relevant criteria for screening of novel yeasts for brewing industry (Nikulin et al., 2020). Maltotriose assimilation has been associated to the presence of *AGT1* sequences in the yeast genome (Anja B., 2019). For *S. pastorianus*, sequences encoding a maltotriose transporter with high similarity with genes involved in maltose transport were identified by Salema-Oom et al. (2005).

In general, dextrins are not fermented by the conventional brewer' yeasts (Hammond et al., 1995; Krogerus et al., 2017). Excepted by *S. cerevisiae* var. *diastaticus*, which demonstrates the ability to metabolize these more complex compounds, being employed in the production of low-calorie beers, as they great reducer of sugar present in the beverage (Krogerus et al., 2017). Park et al. (2014) obtained genetically modified strains capable of expressing glucoamylase gene (*GAM1*) and, consequently, degrading dextrin from the wort and generating low calories beers. Techniques using hybridization have also been applied to obtain strains able to metabolize more complex sugars from wort, thereby increasing ethanol yield in the beverage and reducing carbohydrate concentration (Krogerus et al., 2017).

2.2.2. Free Amino Nitrogen

Efficient nitrogen uptake from the wort is also an important property of yeasts for beer production (Michel et al., 2016). The nitrogen sources available for

microorganisms are called Free Amino Nitrogen (FAN) (Hill et al., 2019). FAN type and concentration interfere in the yeast viability and vitality, in addition to its fermentative efficiency, which impact on beer quality and stability (Lekkas et al., 2009; Stewart et al., 2018). It is also known that different combinations of FAN influence the beer aroma profile (James & Stahl, 2014).

Beer wort consists of a complex mixture of nitrogen molecules assimilable and non-assimilable by microorganisms (Patterson & Ingledew, 1999), which composition varies according to the grain type used (Hill et al., 2019). Approximately 70 % of FAN are generated during malting (Burger & Schroeder, 1976). Amino acids, ammonium ions and small peptides are nitrogenous compounds that can be metabolized (Hill et al., 2019). Their consumption depends on nitrogen catabolic repression (NCR) or cytoplasmic membrane sensors (Ssy1p-Ptr3p-Ssy5) (Crépin et al., 2012). The most part of nitrogen acquired by yeasts come from amino acids, which results in faster growth (Lekkas et al., 2009). Nineteen of the 20 common amino acids can be found in wort, which makes possible the production of up to 400 dipeptides and more than 8,000 tripeptides (Macwilliam et al., 2017). Several transporters for amino acid uptake have been identified including non-specific ones such as general permeases (GAP) and amino acid-specific transporters (Regenberg et al., 1999).

Small peptides can also be used as a nitrogen source by yeasts. It is possible due to their ability to produce extracellular enzymes, facilitating the release of assimilable nitrogen sources (Lekkas et al., 2009). These compounds are used by ale and lager strains, and their uptake occurs simultaneously with amino acids (Hill et al., 2019). Therefore, only 40 % of the peptides present in the wort are generally metabolized by the microorganisms, and these can influence the beer's properties, such as foam stability (Stewart et al., 2018).

2.2.3. Sensorial compounds from yeast metabolism

Besides converting sugars into ethanol and carbon dioxide, brewer' yeasts also must contribute to beer sensorial quality (Saerens et al., 2008). Higher alcohols, esters, sulfur-containing compounds and vicinal dicetones are examples of compounds generated by the yeast metabolism (Figure 2), which only impacts on

sensorial profile if present in amounts above a certain concentration (perception threshold) (Ocvirk et al., 2017) (Table 1).

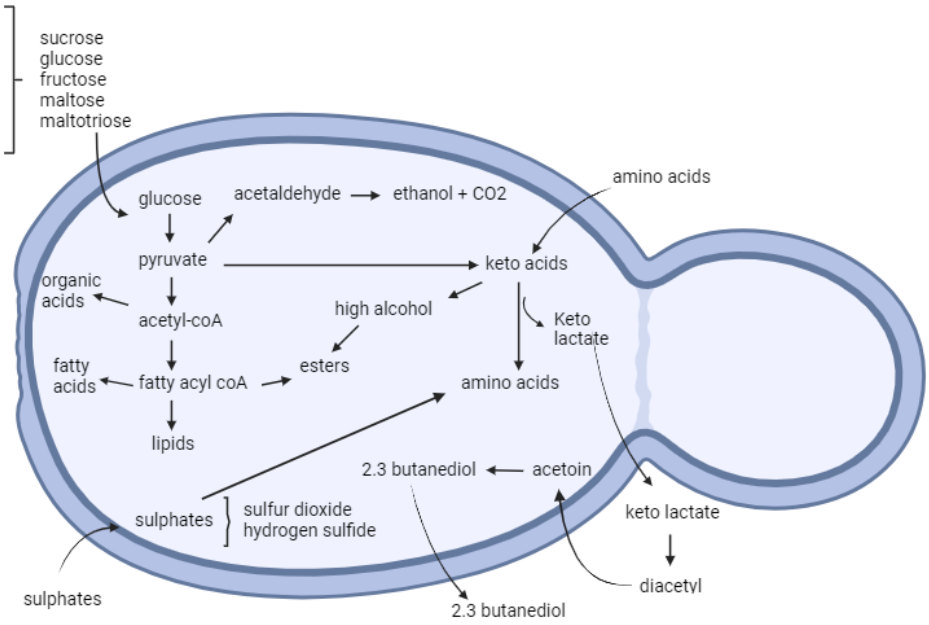


Figure 2. Flavor production from yeast metabolism. After transporting sugars and nutrients into the cell, yeast cleaves sugars into glucose molecules and converts them into alcohol and carbon dioxide. In addition, the pyruvate generated as an intermediate in this process can be converted into organic acids, which impart aroma to the beverage. A variety of sensory compounds are also synthesized, including esters, higher alcohols, diacetyl and sulphur compounds. The regulation of the production of these compounds is influenced by factors such as the composition of the wort, temperature and yeast type.

Table 1. Sensory compounds produced by yeasts and their perception thresholds

Compound	**Group**	**Effect on beer quality**	**Threshold (mg/L)**	**Reference**
N-propanol	Higher alcohols	Bitterness	25.0	Sun & Xiao (2018)
Isobutyl alcohol	Higher alcohols	Alcohol	75.0	Sun & Xiao (2018)
Isoamyl alcohol	Higher alcohols	Alcohol, banana, sweetish	75.0	Sun & Xiao (2018)
β-Phenyl ethanol	Higher alcohols	Roses, sweetish, perfumed	75.0	Sun & Xiao (2018)
Ethyl acetate	Esters	Fruity, solvent-like	21-30.0	Verstrepen et al. (2003)
Isoamyl acetate	Esters	Banana, pear	0.6-1.2	Verstrepen et al. (2003)
Ethyl caproate	Esters	Apple, aniseed	0.17-0.21	Verstrepen et al. (2003)
Ethyl caprylate	Esters	Apple	0.3-0.9	Verstrepen et al. (2003)
Phenyl ethyl acetate	Esters	Roses, honey, sweet	3.8	Verstrepen et al. (2003)
Diacetyl	Vicinal Diketones	Buttery	0.1-0.2 (lager) 0.1–0.4 (ales)	Krogerus et al. (2013)
H_2S	Sulfur compounds	Rotten eggs	0.01	Duan et al. (2004)

Sulfur dioxide (SO2)	Sulfur compounds	Burnt matches	0.025	Landaud et al. (2008)
Methanethiol (MTL)	Sulfur compounds	Cooked cabbage; putrid	2	Ferreira & Guido (2018)
Dimethyl sulfide	Sulfur compounds	Cabbage, corn, onion, blackcurrant	30	Landaud et al. (2008)
Dimethyl disulfide	Sulfur compounds	Cooked cabbage, onion	3-50	Landaud et al. (2008)

More than 90 esters have been described in beer; ethyl acetate, ethyl caproate, ethyl caprylate, isoamyl acetate, isobutyl acetate, ethyl phenyl acetate and ethyl octanoate stand out (Viejo et al, 2019). This group is the most important aromatic compounds in the beverage and even small concentrations can be realized by the consumers (Pires et al., 2014; Xu et al., 2017). Some esters are more easily diffused across the cytoplasmic membrane due to their higher solubility in lipids, such as acetate esters, which are quickly realized in sensory tests (Humia et al., 2019). Their synthesis comes from organic acids and higher alcohols (Ocvirk et al., 2018) and is catalyzed by different enzymes, especially alcohol acetyltransferases (AATs) (Humia et al., 2019). Different conditions can reduce the enzyme activity, such as strain, oxygen and unsaturated fatty acids contents (Hiralal et al, 2013; Iorizzo et al., 2021).

Ester production depends on mainly two factors: i) precursors availability; and ii) the roll of enzymes produced by the yeast (Verstrepen et al., 2003). Pitching rate, temperature, pressure, nitrogen concentration, sugar content in beer wort and dissolved oxygen are factors that also influence the composition of esters of the beverage (Verstrepen et al., 2003). In this sense, supplementing the wort with essential nutrients for yeast growth consists of an interesting strategy to improve the ester production (Hiralal et al., 2014). Interestingly, yeasts also produce esterases which play an important role in beer maturation, when yeast metabolism is decreased due to low temperatures used in this step (Pires et al., 2014; Humia et al., 2019).

Several studies have evaluated different strategies to modulate ester production by yeasts. Changing the expression of *ATF1* and *ATF2* genes encoding alcohol-acetyltransferases was efficient in modulating ester production, reinforcing that their high expression and deletion affect ethyl acetate and isoamyl acetate production during beer fermentation (Verstrepen et al., 2003). Engineering a commercial brewer's yeast resulted in increased *ATF1* gene expression, promoting higher ethyl acetate production (Dong et al., 2019). The expression of *ATF1* gene and deleting BAT2 gene (encoding branched-chain amino acid aminotransferases) resulted in improved ethyl acetate production and reduced higher alcohols, respectively (Zhang et al., 2013).

Higher alcohols, also generated during yeast metabolism, are the most abundant aromatic compounds in beer (Pires et al., 2014). N-propanol, isobutanol,

2-methylbutanol, 3-methylbutanol and 2-phenethylethanol are the ones found in high concentrations (Ocvirk et al., 2017). In general, they come from the Ehrlich Pathway, in which amino acids assimilated from beer wort provide the α-keto acid for higher alcohols synthesis (Pires et al., 2014). Shortly, amino acid undergoes transamination by enzymes catalyzing amino group transfer to its respective α-keto acid, resulting in a new amino acid. In this way, the remaining α-keto acid is decarboxylated and subsequently reduced into a higher alcohol by alcohol dehydrogenases (Ehrlich, 1907).

Sulfur-containing compounds produced by yeasts also influence the beer sensory profile (Ferreira & Guido, 2018), such as hydrogen sulfide, sulfur dioxide, dimethyl sulfide (DMS) and dimethyl disulfide (Ferreira et al., 2012). These by-products present low perception thresholds, representing significative problems for the sensorial quality of the beverage. Nevertheless, some of them can contribute positively to lager beers, for example (Landaud et al 2008). Hydrogen sulfide (H_2S) is tolerated at low concentrations (0.01 mg/L). Factors such as yeast strain, oxygen concentration and the presence of heavy metals can affect its production (Landaud et al 2008). Furthermore, sulfur-containing amino acids, for example cysteine, also increases its synthesis (Duan et al., 2004). The reduction of hydrogen sulfide production by beer-producing yeasts have been evaluated by several studies in last few decades. Omura et al. (1994) modified brewer's yeast strains to express constitutively *MET25* gene, which encodes for enzymes related to sulfhydrylation of H_2S. These reactions promote H_2S depletion, eliminating it from the beverage. Sulfur dioxide has a higher perception threshold than hydrogen sulfide; in addition, it can bind to carbonyls, impacting even slightly on beer sensory profile, by poor aging the beer (Van Haecht & Dofour 1995). Dimethylsulfide (DMS) can also be produced by brewing strains, although upstream steps can also contribute to its generation. Interestingly, yeasts can reduce dimethylsulfoxide (DMSO) present in beer wort converting it into DMS, which provides cooked corn flavors when concentrations above the perception threshold (Ferreira & Guido, 2018).

Vicinal dicetones, including diacetyl (2,3-butanedione), corresponding to another compound group related to beer quality (Ferreira et al., 2012). Its production is related to amino acid valine pathway (Krogerus et al., 2013), where α-acetolactate is generated as an intermediate product and secreted. Probably, it constitutes a strategy for protection of the cell against the action of carbonyl

compounds. Furthermore, it can undergo oxidative decarboxylation, generating diacetyl (Krogerus et al., 2013). In the later stages of fermentation, brewer's yeasts are able to reabsorb the diacetyl produced in the beer (Ferreira et al 2012), from the reduction of diacetyl into acetoin, generating 2,3-butanediol, which presents higher perception thresholds (Mosher & Trantham, 2021). These reactions are catalyzed by specific alcohol dehydrogenases and diacetyl reductases, and have been most described for lager yeasts (Bamforth et al., 2004). Genetically modified strains have been obtained in order to modulate diacetyl production (Gibson et al., 2017). Mutations in *ILV2* gene, which encodes for an α-acetohydroxyacid synthase, have been proposed to reduce the precursor concentration related to diacetyl synthesis (Gjermansen et al., 1988; Li et a., 2002; Shi et al., 2016). Increased expression of *ILV5* and *ILV3* genes, which encode for enzymes promoting α-ketolactate reduction, has also been evaluated (Villa et al., 1995). Shi et al. (2016) investigated the effect of *BDH1* gene overexpression, which encodes for diacetyl reductase, demonstrating a decreased diacetyl concentration in beer.

Some brewing strains produce phenolic compounds from precursors in the beer wort through the decarboxylation of ferulic acid, *p*-coumaric acid or cinnamic acid (Michel et al., 2016) into 4-vinylguaiacol and 4-vinylphenol, for example (Lentz, 2018). For decarboxylation of wort intermediates, yeasts require two enzymes encoded by *PAD1* and *FDC1* genes (Gallone et al., 2016). *PAD1* encodes for phenylacrylic acid decarboxylase, which catalyzes the synthesis of the cofactor required for ferulic acid decarboxylase activity, encoded by *FDC1* (Mukai et al., 2014; Lentz, 2018).

However, phenolic compounds production is considered an undesired effect for most part of brewer' yeasts. Strains traditionally used are POF^-, since they have lost genes encoding for enzymes related to precursors decarboxylation (Gallone et al., 2016). Mukai et al. (2014) evaluated the gene sequences of top and bottom fermenting yeast to assess the integrity of *PAD1* and *FDC1* genes; nonsense mutations in both genes were observed. In addition, nonsense mutation and frameshift mutation were observed for *FCD1* gene in bottom fermentation yeasts.

2.3. Flocculation

When the fermentative process is concluded, it is important to remove easily the yeasts from the beverage, in order to generate a clear beer with low microbial load. It can be obtained through flocculation, one of the most important properties of brewing microorganisms (Speers, 2016; Kayacan et al., 2020), and represents a significative cost reduction, since less energy is required for beer clarification. In fact, decantation can be improved by low temperatures or centrifugation; however, flocculation significantly optimizes the overall process (Stewart et al., 2018).

Shortly, flocculation consists of the reversible adhesion of yeasts, in an asexual manner, in which flocs composed of thousands of cells are formed and quickly come out of suspension (Kerr et al., 2018). Several factors can influence on aggregates formation, such as genetic properties and environmental conditions (Stewart, 2018). It is worth mentioning that this mechanism is considered distinct from the other aggregation strategies identified in *Saccharomyces* species (Kerr et al., 2018). Flocculation is an important adaptive advantage for the yeast, since cell autolysis inside flocs can provide nutrients to remaining yeasts in environments where nutrients are limited (Stewart et al., 2018). Several hypothesis have been suggested to explain how cells bind together during flocculation. The most reported mechanism addresses the presence of lectin class proteins on the cell surface, called flocculins. They are firstly encoded by *FLO1* gene, located on chromosome 1 (Miki et al., 1982). Although *FLO1* is the gene most studied, *Saccharomyces* has other correlated genes, such as *FLO5*, *FLO9*, and *FLO10* which also encode for proteins related to cell-cell adhesion (Nayyar et al., 2017).

Flocculin expression regulation is complex due to the many factors involved in FLO gene family transcription (Teunissen et al., 1995). Furthermore, the number of yeast generations can also interfere in the gene regulation (Verstrepen et al., 2003). Moreover, the number of gene repetitions, such as *FLO1*, interferes with the degree of flocculation (Smukalla et al., 2008).

In addition to all the genetic factors, flocculation depends on the collision of the cells each other (Verstrepen et al., 2006). Conditions that increase hydrophobicity or reduce negative charges on cell surface can intensify the process, since they increase the possibilities of cell interactions (Verstrepen et al., 2003). In this context, yeasts can be classified according to their flocculation capacity, being

described as highly, moderately or non-flocculent (Mehta et al., 2020). Its classification considers the methodology proposed by the American Society of Brewing Chemist (ASBC), known as Helm Test (ASBC, 2011).

The cell aggregation induced by flocculins results from their binding to mannose residues from adjacent cells, which may or not be a flocculent strain (Miki et al., 1982; Stewart et al., 2018). As mannose is present in all yeast cells, the critical factor for cell flocculation is the presence of flocculins. Besides that, calcium is also required, as it activates lectins present on cell wall surface (Verstrepen et al., 2003). Therefore, the presence of mannose in substrate is an inhibitory factor for flocculation process, since they can bind to flocculins, preventing cell aggregation. It is important to highlight that for some strains other sugars can also repress the flocculation. For example, strains known as NewFlo have their flocculation process inhibited in the presence of glucose, sucrose and maltose. In *S. pastorianus*, *LG-FLO1* gene, not found in ale strains, was identified; its expression is responsible by the NewFlo phenotype, in which flocculation is regulated by mannose and glucose (Verstrepen et al., 2003). Strains regulated only in the presence of mannose are classified as Flo1 phenotype (Stratford et al., 1991; Sieiro et al. 1995).

Ale beer strains are classified as top fermentation due to the interaction of flocs with CO_2 bubbles; therefore, the aggregates are carried to the top of the fermentation tank, forming a visible crown of cells. In contrast, *S. pastorianus*, used in lager beers, are designated as bottom fermentation, as the flocs do not interact with carbon dioxide, remaining at the bottom of fermentation tank (Menezes e Silva, 2019). In beer, yeasts need to flocculate at the appropriate time since they need to remain long enough in contact with the wort. The amount of cells in suspension during primary and secondary beer fermentations affects its flavor profile, fermentation rate, maturation and filtration (Stewart et al., 2018). For high flocculent profile yeasts, sedimentation can occur too quickly creating under-attenuated beers with high residual sugar. In addition, these beers may be more susceptible to microbiological contaminants. On the other hand, delayed flocculation yeasts result in dry beers causing problems in filtration step, as well as off-flavors generation such as yeast flavor (Menezes e Silva, 2019).

2.4. Genomic properties

Genome of microorganisms employed in brewing industry was adapted during the time, after thousands of years of hybridization events combined with the domestication processes (Steensels et al., 2019). For winemaking, strains used in nowadays come from Asian and other strains not yet characterized. Later, they have been intensively used for brewing, being exposed to a continuous domestication process. As result, distinct yeasts were generated, establishing the diversity of strains available today (Davydenko et al., 2020).

Strains can be classified in two groups according to their genetic properties: Beer 1 and Beer 2. The first one is composed of domesticated strains with geographical influences, represented by German, British and American strains. In turn, the second group is recognized by its extensively use for wine production (Gallone et al., 2016). Furthermore, there is also a distinct yeast group not included in both divisions known as Kveik. These strains have been isolated for many years from Norwegian farms, with low level of domestication. Kveik strains have particularities in relation to conventional brewing strains, such as tolerance to higher temperatures and production of high ethanol levels (Preiss et al., 2018).

Brewing yeasts present distinct genomic properties in comparison with wild strains (Davydenko et al., 2020). The most relevant consists of the number of chromosomes: while brewing strains are polyploid or aneuploid wild type strains are haploid or diploid (Steensels et al., 2019), a feature generated after an extensive adaptation process when exposed to stress conditions (Yona et al.,2012). Chromosome duplication is an important evolutionary event in S. cerevisiae as it results in advantages for the yeast in the industrial environment, since mutation events do not necessarily cause changes in phenotype. Moreover, relevant genes can generate more copies; for example, genes coding for abundant carbon sources assimilation in wort, such as maltose (Davydenko et al., 2020; Molinet et al., 2020).

Other modifications occurred in the brewing yeast genome, such as Copy Number Variation (CNV) (Steensels et al., 2019). Gonçalves et al. (2016) investigated copy number variation of the *MAL1* and *MAL3* locus in over 20 brewing strains. The results showed higher copy number of *MAL3* mainly in German strains, in which 15 repetitions were observed. Most of the other strains had more than six copies of this locus in the genome. Copies of *MAL1* were also

identified, although they were present in smaller quantities. Such genetic modifications are visualized mainly in telomeric regions, where genes responsible for adaptations to environmental changes are found. These regions are characterized, for example, by the presence of genes involved in resistance to stress conditions and nutrient uptake (Steensels et al., 2019).

Events such as CNV, associated with whole genome duplication, are involved in relevant changes on *Saccharomyces*-type yeasts (Davydenko et al., 2020). Duplication of gene encoding for alcohol dehydrogenase and multiple copies of hexose transporters from genome duplication contribute to the Crabtree effect. It consists of the ability to obtain energy gain from the fermentative process even in the presence of oxygen. (Hagman et al., 2013).

The extensive use of yeasts for brewing purposes were responsible for the emergence of lager beer strains. The combination of domesticated *S. cerevisiae* (extremely efficient on maltotriose metabolization) with a *S. eubayanus* strain (with high tolerance to low temperatures) gave rise to *S. pastorianus* strains (Libkind et al., 2011; Vrie et al., 2019).

Similarly, these yeasts can be also divided in two groups: Saaz and Frohberg (Gibson et al., 2013). The first group contain large amounts of genes from *S. eubayanus,* which reflects in greater tolerance to low temperatures. In contrast, they are unable to metabolize maltotriose; as result, these strains have low fermentation rates and consequently low ethanol yields. The second group is composed by yeasts really efficient in the utilization of both maltose and maltotriose (Gibson et al., 2013). As these strains are tri- (Saaz) or tetraploid (Frohberg), an origin independent of hybridization events (Dun & Sherlock, 2008) or a single shared hybridization followed by subsequent events can explain the emergence of these groups (Okuno et al., 2016). In this sense, the combination of a *Saccharomyces* haploid strain with a *S. eubayanus* diploid strain might have originated the triploid pattern (Saaz). In a second independent hybridization event, a *S. eubayanus* diploid strain would have combined with a *S. cerevisiae* haploid, giving rise to tetraploid strains (Frohberg) (Dun & Sherlock, 2008). Meanwhile, another theory based on at least one shared hybridization event has been proposed (Gorter de Vries et al., 2019).

Currently, beers produced with lager strains represent 90 % of worldwide production (Lengeler et al., 2020). However, their diversity is still limited.

Genetically modified strains with improved fermentation performance able to reduce off-flavors have already been developed. Nonetheless, the brewing industry still does not approve the use of this type of strain for large scale production (Gorter de Vries et al., 2019).

2.5. Tolerance to stressful conditions

2.5.1. Cold Temperature

During beer production, yeasts are exposed to low temperatures several times; for example, yeasts can remain at the bottom of the fermentation tank at temperatures ranging from 2 to 11 °C after fermentation (Gibson et al., 2007). Moreover, when acid washing is performed, the process takes place at temperatures between 2 to 4 °C (Bleoanca & Bahrim, 2010). It promotes reduced fluidity of cytoplasmic membrane, resulting in decreased transport capacity. A reduction in hydrophobic interactions between peptides is also affected, resulting in protein denaturation (Gibson et al., 2007). In addition, low temperatures can impair protein folding, as well as increase conformational instability causing protein denaturation too. In general, gene transcription can be affected as result of increased interactions between DNA strands (Vicent et al., 2015). Besides that, the translation can be impaired due to secondary structures in RNA molecule (Sahara et al., 2002).

All these situations can promote cold shock in yeasts. For *S. cerevisiae*, this phenomenon can occur at temperatures below 20 °C (Gibson et al., 2007). In contrast, lager yeasts are adapted to growing at reduced temperatures (Paget et al., 2014). Differences in lipid content of cytoplasmic membrane is considered an adaptation mechanism to reduced temperatures (Rodriguez-Vargas et al., 2020). In this condition, brewer's yeast membranes have higher proportions of unsaturated fatty acids, such as oleic and palmitoleic acids, which increases its fluidity (Krogerus et al., 2017). Mitochondrial DNA of lager strains plays an important role in their tolerance to low temperatures (Li et al., 2019). *S. pastorianus* presents higher proportions of mitochondrial DNA from the *S. eubayanus* parental strain, which is characterized for fermenting at low temperatures (Rainieri et al., 2008). Studies about the role of mitochondrial DNA in synthetic (*S. cerevisiae* x *S. eubayanus*) and industrial (*S. pastorianus*) hybrid strains revealed that the tolerance

mechanism corresponds to the parental mitochondrial DNA providing ways to modulate this property in lager strains (Baker et al., 2014).

Lager strains can ferment faster than *S. cerevisiae* at lower temperatures. The activity of maltose transporters in lager and ale strains are similar at 20 °C; however, transporters from lager strains are five times more active at 0 °C (Vidgren et al., 2010). Therefore, lager strains have specific maltose transporters, which results in higher fermentation efficiency at low temperatures. Furthermore, differences in cold tolerance can be observed for the different groups of lager strains (Monerawela & Bond, 2017). For example, Saaz strains are more tolerant due to the higher amount of genome derived from the cryotolerant parental strain *S. eubayanus* (Gibson et al., 2015).

In last few years, efforts to obtain novel yeast strains able to ferment at low temperatures combining cryotolerant with *S. cerevisiae* strains has been done (Nikulin et al., 2018). Krogerus et al. (2015) obtained hybrids from *S. eubayanus* and *S. cerevisiae* able to ferment at low temperatures, flocculating properly and using maltotriose. Nikulin et al. (2018) recreated low temperature tolerant yeasts for lager beer from hybridization techniques, combining *S. cerevisiae* with non-*Saccharomyces*. The authors highlighted this methodology as an effective way for generating new strains resistant to several stress conditions commonly observed during brewing.

2.5.2. Ethanol content

For most beers, up to 3 to 6 % alcohol can produced during fermentation; however, when using a high-gravity wort, more than 10 % can be achieved (Gibson et al., 2007). For most of yeast strains, ethanol concentrations higher than 6 % are enough to inhibit the cell viability (Bleoanca & Bahrim, 2013). It is related to changes on glucose transportation as well as other nutrients, influencing metabolic activity and causing reduction in cell size (Leão & Uden, 1983; Canetta et al., 2005). Ethanol accumulation can also cause acidification of the cytoplasm (Teixeira et al., 2009). Moreover, ethanol can inhibit relevant enzymes related to the glycolytic pathway (mainly hexokinases), promoting the generation of reactive oxygen species, harming the correct mitochondrial functionality (Casey & Ingledew, 1986; Gibson et al., 2007).

In addition, ethanol promotes damage to mitochondrial DNA, generating petites mutants; it is observed mainly after many yeast reuse cycles by the brewing industry (Gibson et al., 2007). Chi & Arneborg (1999) demonstrated that strains more resistant to ethanol due to mitochondrial mutations present high proportions of ergosterol, high levels of phosphatidylcholine, higher incorporation of long-chain fatty acids and higher proportions of unsaturated fatty acids in mitochondrial membrane.

High ethanol concentrations also change the cytoplasmic membrane structure increasing its permeability; consequently, dissipation of electrochemical potential is observed (Teixeira et al., 2009). Changes in ergosterol or phospholipid composition and levels can prevent this type of effect (Vamvakas & John Kapolos, 2020). Adding inositol in the medium has already been shown to increase the viability of *S. cerevisiae* strains exposed to ethanol, reducing membrane permeability (Furukawa et al., 2004). Finally, lower tolerant strains present higher proportions of phosphatidylethanolamine, evidence that changes in cell membrane can be related to ethanol exposure (Lairón-Peris et al., 2021). In general, the lower fluid is cell membrane, the more resistant to ethanol (Ishmayana et al., 2017).

Problems due to intracellular environment acidification can be solved by increasing activity of H^+ATPases, named Pma1p and Pma2p, which carry out proton transport, maintaining cell pH controlled (Ferreira et al., 2001; Teixeira et al., 2009). Furthermore, under ethanol stress conditions, an increasing expression of genes encoding these proteins is observed (Aguilera et al., 2006; Lee et al., 2016). Heat shock genes are also activated during stress caused by ethanol (Costa 1993; James et al., 2003); *HSP12, HSP26, HSP30* and *HSP42* genes are overexpressed during ethanol exposure (Vamvakas & Kapolos, 2020). The activation of this regulation system can promote the expression of chaperones, which act preventing protein aggregation (Foster et al., 2021). Another mechanism to tolerate ethanol stress consists of intracellular trehalose accumulation. Foster et al. (2021) demonstrated that Kveik strains can tolerate higher concentrations of ethanol in comparison with most conventional brewer strains due to the significative intracellular trehalose accumulation, protecting and stabilizing proteins. In addition, trehalose also acts by reducing membrane permeability, increasing the effects caused by ethanol stress (Gibson et al., 2007).

Molecular mechanisms involved in ethanol tolerance have been evaluated to explain stress condition tolerance (Vamvakas & Kapolos, 2020). Genome sequencing results revealed single nucleotide mutations, copy number variation, and ploidy changes in ethanol tolerant strains. It could be related to different evolutionary mechanisms that play an important role on yeast survival (Voordeckers et al., 2015). Gene sequences related to intracellular organization, transport and transcriptional machinery have been related to ethanol resistance; in addition, FPS1 gene encoding for an aquaglyceroporin related to glycerol efflux control was described; its expression could also reduce ethanol accumulation inside the cells (Teixeira et al., 2009).

2.5.3. Osmotic stress

During beer production, yeasts are frequently exposed to osmotic stress conditions. For example, acid washing step increase H^+ ions concentration (Gibson et al., 2007). After wort inoculation, yeasts are also subjected to stressful conditions due to high concentrations of sugars present there. Considering high-gravity beer wort, this effect is even more intensified (Puligundla et al., 2011).

Recently, several breweries have been employing high-gravity wort for beer production as it increases the brewery efficiency (Piddocke et al., 2009). In this scenario, large amounts of adjuncts must be employed; therefore, a lack of nutrients can represent a problem for the microbial physiology (Kincla et al., 2021). In general, high-gravity wort results in high ethanol concentrations (up to 16 %). In this case, the yeasts must be ethanol tolerant, present good physiological conditions and be adapted to assimilate sugar sources in high-density wort (Giannakou et al., 2020).

Osmotic stress causes a reduction cytoplasmic membrane fluidity and damages to microbial DNA. Under conditions of intense osmotic stress, *S. cerevisiae* cell size can be reduced, generating damages in its DNA (Ribeiro et al., 2006). Furthermore, under osmotic stress conditions, yeasts can produce acetate esters in high concentrations, providing off-flavors as a solvent for the beverage (Dekoninck et al., 2012). Commercial lager strain Weihenstephan 34/70 produces higher concentrations of ethyl acetate and isoamyl acetate besides a reduced

specific growth rate and prolonged Lag phase under osmotic stress conditions (Piddocke et al., 2007).

Another undesirable effect related to osmotic stress conditions consist of reduced beer foam stability (Stewart et al., 2010). Higher proportions of Proteinases A secreted by yeasts in this context causes a reduction in hydrophobic polypeptides amount in the wort, impacting negatively foam stability (Brey et al., 2002). Hao et al. obtained lager yeast mutants no encoder for Proteinase A and observed higher foam stability and no changes in the sensory profile of the beverage.

Gibson et al. (2007) demonstrated that brewer's yeasts are more resistant to osmotic stress during stationary phase compared to exponential phase. It can be explained by the presence of Stress Responsive Elements during the stationary phase (Bleoanca et al., 2003). Oomuro et al. (2018) evaluated the effect of intracellular accumulation of S-adenosylmethionine (SAM), which is involved in the glycolytic pathway, during the growth of lager yeasts in high-gravity wort. The results demonstrate that SAM increases fermentation rates, optimizing the brewing production. Trehalose accumulation, H^+ATPases activity, cytoplasmic membrane optimization and vacuole activity are considered strategies used by yeasts for survival in osmotic stress environments (Gibson et al., 2007; Gao et al., 2010). For *S. cerevisiae,* the accumulation of compatible solutes plays an important role on osmotic potential regulation, preserving it's the physiological integrity of the yeast (Gibson et al., 2007). James et al. (2007) observed a greater genomic plasticity in lager strains when it was applied in sequential fermentations, both in high-gravity wort and high temperatures. It reinforces their ability to adapt to stress conditions through gene plasticity.

Trehalose and glycerol can also contribute to protecting cell from osmotic stress. Glycerol acts by performing an equilibrium in concentrations of the cell's internal and external environment. In turn, trehalose improves protein stability and cytoplasmic membrane maintenance (Zhuang et al., 2017). Trehalose accumulation is directly related to the increase in wort sugar concentration (Majara et al., 1996).

2.5.4. Other factors

Yeasts are exposed to several other stress factors during beer production such as low pH, oxidative stress and the presence of hops (Sanchez et al., 2012,

Gibson et al., 2020). As mentioned above, these factors can be intensified when these microorganisms are exposed to successive fermentations during yeast reuse, as well as during yeast storage (Gibson et al., 2007). For *S. cerevisiae*, different stress conditions activate a general stress response (GSR), which acts as an evolutionary mechanism, providing a quick response to environmental changes in a non-specific manner (Ruis & Schuller, 1995). The expression of more than 200 genes is observed when the system is induced. Mechanisms specific to each stress condition can also be activated when yeast cells are submitted to stressful conditions (Gasch et al., 2017).

Before yeast pitching, the wort must be aerated. It is necessary since yeast needs to activate the respiratory mechanism to increase cell biomass and to carry out the fermentation efficiently. In addition, oxygen is important for ergosterol and unsaturated fatty acids synthesis (Gibson et al., 2007). During the aerobic metabolism oxygen is also responsible for reactive oxygen species (ROS) generation, which cause several cellular damages, affecting negatively DNA, lipids, sugars and proteins (Verbelen et al., 2009). Defense mechanisms against ROS include the production of peroxidases, catalases, and superoxide dismutases. Antioxidants such as gluthatione and thioredoxin and vitamins also help to protect yeast cells (Grant et al., 1996; Verbelen et al., 2009). Furthermore, the yeasts have a transcription factor known as Yap, which activates the transcription of various proteins to protect against oxidative stress (Estruch, 2000).

Clarkson et al. (1991) demonstrated the behavior of lager and ale yeasts removed from the anaerobic environment and exposed to an oxygen condition. The authors identified increased CuZn-superoxide dismutase activity and a reduction of 7 % in cell viability. Verbelen et al. (2009) evaluated the effect of wort oxygenation on brewer's yeast metabolism and oxidative stress response, demonstrating a trehalose accumulation and induction of *HSP12, SSA3, PAU5, SOD1, SOD2, CTA1* and *CTT1* genes involved with oxidative stress response.

pH reduction is also a stress factor for brewer's yeast. Several changes can be observed after expected pH reductions during beer fermentation, such as altered gene expression, interference in flavor production and reduced viability (Imai & Ohno, Kapteyn et al., 2001; Gibson et al., 2007). H^+ATPases activity is essential for yeast tolerant to low pH conditions (Carmello et al., 1996).

Finally, yeasts must also be able to grow in the presence of hops (Michel et al., 2006), considered an antimicrobial agent in beer by dissipating the electrochemical potential of the cytoplasmic membrane (Bergsveinson et al., 2015). Hazelwood et al. (2020) studied the mechanisms involved in hop tolerance. It was shown that yeasts may be able to alter the cell wall to promote iso-alpha-acid binding, which prevents these compounds from becoming free in the cytoplasm. Yeasts can also act by driving antimicrobial compounds out of the cell. The third and last mechanism described is the accumulation of these molecules inside the vacuole, protecting the cell from their antimicrobial effects.

2.6. Prospecting new yeasts for innovative beers

The current demand for innovative products has increased the microorganism prospecting for use in beer production (Table 2) (Gibson et al., 2020). In this context, beverages with lower alcoholic and calorie content with improved sensorial profile are desirable (Canonico et al., 2021; Puligundla et al., 2021). *Torulaspora delbrueckii*, *Lachancea thermotolerans* and *Pichia kluyveri* are the most common species related to this effort (Canonico et al., 2017; Gibson et al., 2017, Sannino, et al., 2019).

For alcohol-free or low alcohol content beer production, thermal or membrane processes, as well as control of fermentation time are promising strategies (Bellut et al., 2018; Capece et al., 2020). In this case, beer may be perceived as sweeter due to an incomplete sugar metabolization (Bellut et al., 2018).

Strains with reduced ability to ferment maltose and other abundant sugars in the wort, resulting in in lower alcohol production, have been evaluated as an alternative (Jonhansson et al., 2021; Puligundla et al., 2021). Besides providing the expected low alcohol content, these strains don't impact the sensorial beer quality, in opposition to conventional methods for this purpose. Furthermore, it has low cost as uses the same facility, not requiring significative investments (Capece et al., 2020).

Currently, *Saccharomycodes ludwigii* has already been employed commercially for low-alcohol beer production (Puligundla et al., 2021). However, few studies have been conducted to prospect new microorganisms (Bellut et al., 2018). Bellut et al. (2018) investigated the potential of *Hanseniaspora valbyensis*,

H. vineae, *T. delbrueckii*, *ZygoSaccharomyces bailii* and *Z. kombuchaensis* for brewing application. All the strains evaluated showed high viability in the wort, as well as sensory properties similar to the control, which demonstrates their potential for application in brewing.

Johansson et al. (2021) also evaluated new yeasts for low alcohol beer production. Autochthonous yeasts from sourdough bread were evaluated for their ability to tolerate stressful conditions commonly observed during beer production. A *P. fermentans* strain resulted in a promising sensory profile for Belgian wheat beer with reduced alcohol content. Moreover, *Kazachstania servazzii*was able to tolerate stress conditions and produce low alcohol content, besides a neutral sensory profile, potentially used for lager beer production.

Besides lower alcoholic beers, lower-calorie beverages have been created in last years. Park et al. (2014) obtained genetically modified yeasts able to express *GAM1* gene for glucoamylases, which confers dextrin degradation and, consequently, reduces the beer calorie content. Adding amyloglucosidase and diastase in the beverage is also an alternative for reducing the caloric content. However, it is considered a high-cost methodology, while genetically modified strains are not yet well accepted by the beer market (Troilo et al., 2019). In this context, non-*Saccharomyces* yeasts producing amylases for converting complex sugars, such as dextrins, into fermentable compounds has been proposed. It reduces the calorie beer content, meeting demand for healthy lifestyles (Puligundla et al., 2021). *SchizoSaccharomyces pombe* was demonstrated as a promising yeast for this effort (Troilo et al., 2019).

Non-conventional yeasts can also generate more complex sensory profile, generating innovative beverages for the brewing industry (Gamero et al., 2020). The yeasts can perform individually, assuming the complete fermentation of the beverage, or in co-fermentations, acting together with a conventional yeast (Revasio et al., 2018). Moreover, they can be employed in sequential fermentations, being added after the addition of conventional yeasts during the beer maturation, for example (Holt et al., 2018). Ravasio et al. (2018) evaluated the effect of co-fermentation of a commercial lager strain *Weihenstephan* 34/70 and *Wickerhamomyces anomalus*. The results showed an increased concentration of fruity flavors, the same that observed for *T. delbrueckii*. Moreover, the strains were

efficient in sugar conversion, not being affected by antimicrobial properties of hops neither ethanol concentration (Michel et al., 2016).

Non-*Saccharomyces* yeasts can improve the sensory beer profile due to their ability to encode enzymes that transform hop compounds, such as monoterpenes. It can release sensory compounds such as linalool and limonene, promoting desirable flavors (Michel et al., 2016). Other species can produce phenolic compounds through the activity hydroxynamate decarboxylases that modify intermediate compounds from hops and grains, such as ferulic acid and p-coumaric acid (Holt et al., 2018).

Table 2. Non-conventional yeasts used in brewing

Yeast	Properties	Reference
Brettanomyces	Production of ethyl acetate, ethyl caprate, ethyl caprylate and ethyl lactate, high ethanol yield, tolerance to low pH, production of volatile phenols (4-ethylphenol and 4-ethylguaiacol), and β-glucosidase activity.	Colomer et al. (2018); Iorizzo et al. (2021)
Lachancea thermotolerans	Production of low acetic acid and high lactic acid.	Domizio et al. (2016); Iorizzo et al. (2021)
Saccharomycodes ludwigii	Low production of ethanol, production of ethyl acetate, isoamyl acetate and 4-vinylguaiacol, low production of diacetyl.	Francesco et al. (2015); Iorizzo et al. (2021)
Torulaspora delbrueckii	Significant production of 2-phenylethanol, n-propanol, iso-butanol, amyl alcohol, and ethyl acetate, ability to convert hop monoterpene alcohols into linalool.	Basso et al. (2016)
Kluyveromyces marxianus	Production of high levels of the rose-like flavour phenylethanol and 2-phenylethyl acetate.	Gibson et al. (2017)
ZygoSaccharomyces rouxii	Low ethanol and production of ethyl acetate, amyl alcohols, and isoamyl alcohols.	Sohrabvandi et al. (2009); Francesco et al. (2015), Methner et al. (2019)
Hanseniaspora vineae	Low- alcohol and high esters production.	Larroque et al. (2021)

Wickerhamomyces anomalus	Production of ethyl propanoate, phenyl ethanol, 2-phenylethyl acetate, and ethyl acetate.	Basso et al. (2016)
Kazachstania servazzii	Low alcohol production, and neutral sensory profile.	Johansson et al. (2021)

The yeasts used in the brewing process are quick to ferment the wort and this is of great interest to the industry. However, it is important to point out that fermentation is still a barrier in the production stages of the beverage due to the long time involved. In addition to fermentation efficiency, it is also important to emphasize the sensory quality required during production. Although conventional yeasts have high fermentation rates and do not produce off-flavors, they have been extensively studied and subjected to optimization processes, such as laboratory domestication and genetic modifications to support the industry and the modernization of the production process. The presence of increasingly potent yeasts on the market is an expectation of the brewing industry, where they can show increased tolerance to factors such as alcohol and osmolarity, as well as the production of volatile compounds different from the conventional ones. It is also important to mention the preference of consumers, who are becoming increasingly demanding. Alcohol-free beers with an innovative sensory profile are examples of trends in the brewing sector. In this sense, it is worth noting that non-*Saccharomyces* yeasts are increasingly being evaluated as a promising alternative for these preferences.

3. MATERIAL AND METHODS

3.1. Coffee sampling

First of all, mature coffee beans (Topázio Amarelo, Colombiano and Catuaí Vermelho varieties) from 2020 harvest, coffee beans from dry fermentation and liquid samples from spontaneous wet fermentation process were collected from farms in São Sebastião do Paraíso, State of Minas Gerais, Brazil (Table 3). The samples were packed in plastic bags and immediately sent to the Biotechnology and Biodiversity for the Environment Laboratory. Before the isolating step, the coffee beans were washed with water and added to 0.02 Mm potassium phosphate buffer followed by a ground step in a Polytron™ type homogenizer. Regarding the wet fermentation samples, the liquid samples were collected and transported to the LBBMA in same conditions described above.

Table 3. Samples and isolation conditions

Source	Coffee Variety	Coordinates	Condition
Coffee beans	Topazio Amarelo	21º01’16,8’’S/ 46º54’06,4’’W	15 °C
	Colombiano	20º51’24,6’’S/ 47º03’09,3’’W	15 °C
	Colombiano	20º51’24,6’’S/ 47º03’09,3’’W	20 °C
Coffee beans (dry fermentation)	Catuaí Vermelho	20º51’24,6’’S/ 47º03’09,3’’W	15 °C
Coffee beans (dry fermentation)	Catuaí Vermelho	20º51’24,6’’S/ 47º03’09,3’’W	20 °C
Liquid (wet fermentation)	Catuaí Vermelho	20º51’24,6’’S/ 47º03’09,3’’W	20 °C
	Topázio Amarelo	20º51’24,6’’S/ 47º03’09,3’’W	20 °C

3.2. Yeast isolation

The samples obtained from coffee beans were diluted in 0.02 mM potassium phosphate buffer and plated in Malt Yeast Glucose Peptone Medium (MYGP) (yeast extract (3 g L^{-1}), peptone (5 g L^{-1}), glucose (20 g L^{-1}), malt extract (3 g L^{-1}), supplemented with 0.25 g L^{-1} chloramphenicol). Its pH was adjusted to 5.6 and the plates were incubated at 15 and 20 °C for five days, according to Helson (2009). Regarding the samples obtained from the wet fermentative process, dilution was performed using peptone salt solution followed by plating onto Yeast Extract-Peptone-Dextrose (YEPG) medium (yeast extract (5 g L^{-1}), glucose (20 g L^{-1}), peptone (10 g L^{-1})). The pH was adjusted to 5.6 and the medium was supplemented with 0.25 g L-^{1} chloramphenicol). The isolates were diluted to 10^5 fold dilution and plates were incubated at 20 °C for five days selection was based on phenotypic differences.

3.3. Growth evaluation on different carbon sources

The capacity to grow on different carbon sources of the yeasts were performed according to Marongiu et al. (2015), with modifications. For this, the isolates were grown in test tube with YNB medium (Yeast Nitrogen Base, 0.67 % w/v), added to 20 g L^{-1} glucose or maltose, after standardization to 0.2 optical density at 600 nm ($OD_{600\ nm}$). Therefore, the cultures were incubated at 25 °C at

300 rpm for 24 hours; after, the measure of OD_{600nm} was analyzed. The isolates were also evaluated for growth ability on maltotriose. For this, the cultures were previously grown and inoculated (OD_{600nm}) in YNB medium with 20 g L^{-1} maltotriose. Assays were conducted in microplates and growth evaluation was conducted after 24 hours.

3.4. Tolerance to alcohol, pH, and temperature

The yeast's tolerance to alcohol was evaluated submitting the isolates cultures previously standardized (OD_{600nm}: 0.2) to growth in YPD (10 g L^{-1} yeast extract, 20 g L^{-1} peptone and 20 g L^{-1} glucose) with ethanol at different concentrations: 5, 8, 10 and 15 % (v/v). The cultures were incubated at 25 °C for 7 days and the $OD_{600\ nm}$ measurement was determined every 24 hours. To evaluate the yeast tolerance to different pH values, YPD with pH adjusted to 3.5 and 4.5 with 1.0 M Hydrochloric Acid Buffer Solution was used. The cultures were grown at 25 °C for 7 days and the $OD_{600\ nm}$ measurement was obtained every day. To evaluate low temperature tolerance, cultures were grown in YPD and incubated for seven days at 18 and 20 °C, following the same protocol described above. The control for all the tests was conducted in YPD and incubated at 25 °C.

3.5. H_2S production

The H_2S production was determined after growth on plates containing LA medium (40 g L^{-1} glucose, 5 g L^{-1} yeast extract, 3 g L^{-1} peptone, 0.2 g L^{-1} ammonium sulfate and 1 g L^{-1} lead acetate, 15 g L^{-1} agar), incubated at 28 °C for 10 days (Ono et al., 1991). Black colonies grown on the agar were considered as positive for H_2S production; white or cream colonies, negative for H_2S production; beige colonies, low producer; and, brown colonies were considered as medium producers. A commercial *S. cerevisiae strain* (US-05, Fermentis®) was used as negative control.

3.6. Production of phenolic off-flavors

The production of phenolic compounds by the isolates was performed according to Meier-Dörnberget T. et al. (2018). The isolates were grown in YM

medium (3 g L^{-1} malt extract, 3 g L^{-1} yeast extract, 5 g L^{-1} peptone, 11 g L^{-1} glucose, and 15 g L^{-1} agar), supplemented with 2 ml ferulic acid (5 % diluted in 96 % ethanol), and the plates were incubated for at 24 °C three days. Cultures presenting clove aroma were considered positive. A commercial *S. cerevisiae strain* (US-05, Fermentis®) was used as negative control.

3.7. Taxonomic identification

The yeast isolates selected in the previous steps were identified by partial sequencing of the 26S rDNA gene. For this, the isolates were cultivated in YPD medium at 30 ºC for 24 hours and DNA was extracted as following: 2 mL of each culture was centrifuged and the supernatant was discarded. The pellet was resuspended in tubes containing 400 uL of extraction buffer (20 mL L^{-1} Triton 100x, 100 mL L^{-1} SDS 10 %, 100 mL L^{-1} NaCL 1M, 100 mL L^{-1} Tris 1 M (pH 8), 1 ml L^{-1} EDTA 0,5 M (pH 8)) added to 0.3 g of glass beads. The tubes were shaken for 5 minutes and centrifuged (12,000 rpm). The supernatant was transferred to a new tube and 200 µL of phenol:chloroform:isopropanol (100 µL phenol, 96 µL chloroform, and 4 µL isopropanol) solution were added to the microtubes, followed by gentle agitation (manually inverted about 10 times). The tubes were centrifuged (12,000 rpm, 10 min) and the supernatant transferred to a new flask. After, 300 µL of chloroform was added and the flasks were gently shaken (manually inverted about 10 times). A new centrifugation step was performed (12,000 rpm, 10 min) and the supernatant was transferred to a new tube. Then, 2 volumes (in relation to the supernatant obtained) of ice-cold absolute ethanol and 0.1 volume of potassium acetate (5M) were added to the tube. The mixture was incubated at -80 °C for 30 min and centrifuged (12,000 rpm, 10 min). The supernatant was then discarded and 1 mL of 70 % (v/v) ethanol was added to each tube. A new centrifugation step was performed (12,000 rpm, 5 min) and the supernatant was discarded. After drying, the pellet was resuspended in 30 µL of ultrapure water. The samples were incubated at 37 °C for 15 min. DNA quality was verified in 0.8 % (w/v) agarose gel and the concentration was determined using a Qubit Fluorometer equipment (Life Technologies®). The primers ITS1 (5’ –TCCGTAGGTGAACCTGCGG–3’) and ITS 4 (5’-TCCTCCGCTTATTGATATGC-3’) were used for DNA sequence amplification. Subsequently, the amplicons were analyzed in 0.8 % (w/v) agarose

gel stained with GelRed™ (Biotium); the images were registered in a L-Pix Chemi trans-illuminator (Loccus Biotechnology). The material was sequenced by Fundação Oswaldo Cruz (Fiocruz) and the data obtained was compared with database from GenBank using the BLASTN algorithm available from the National Center for Biotechnology Information website (http: //www.ncbi.nlm.nih.gov).

3.8. Laboratory-scale fermentation test

The isolates were cultivated to characterize the fermentation process on a laboratory scale. For this, the yeasts were previously cultivated in 50 ml of malt extract (12 %), overnight, at 300 rpm and 30 °C. The concentrations of the yeasts were then adjusted to 2.5 x 10^6 cels ml^{-1} using a Neubauer Camera and the isolates were inoculated into 500 ml of wort, in 750 ml glass flasks sealed with metal lids fitted with airlocks. The wort used is of the Pilsen style, acquired from a local brewery. The isolates were maintained for seven days at 20 °C and daily samples were collected for the quantification of sugars and ethanol.

3.9. Pilot-scale beer production

To evaluate fermentation on a bigger scale, a pilot batch of a Cream Ale-style beer was produced. To this end, 30 liters of beer were produced using the US05 yeast (Fermentis®) as the experiment control, and 30 liters using the yeast isolated in this study in co-fermentation with conventional yeast, called the treatment. The control batch was made from 5 kg of Pilsen malt, 30 g of Columbus hops, 30 g of Cascade hops and 30 liters of water. For fermentation, 11.5 g of US05 yeast (Fermentis®) were used (1.0 x 10^{10} viable cells/g). For the treatment, the same recipe was used as for the control and 380 mL of isolate F605 (3 x 10^8 CFU/mL) and 11.5 g of yeast US-05 (Fermentis®) (1.0 x 10^{10} viable cells/g) were used for co-fermentation. The beer was produced using the following steps: 5 kg of malt were milled and mixed with 30 L of boiled water in stainless steel equipment. The mixture was heated (45°C for 15 minutes; 67 °C for 75 minutes; 72 °C for 15 minutes; and finally, 76 °C for 10 minutes) and then boiled for 60 minutes. During this last stage, Columbus and Cascade hops were added after 10 and 45 minutes of boiling, respectively. The wort was then cooled and transferred to a 50-liter conical

stainless-steel fermenter. The yeast cultures were added to the wort at 20 °C and the airlock fermenters were kept at the same temperature for five days. Subsequently, the fermenters were incubated at 2 °C for maturation for 10 days and, finally, the beer was subjected to the addition of CO_2 for carbonation.

3.10. Beer evaluation

After the brewing process (treatment and control), pH, alcohol content and Soluble Solid Content (SSC) (° Brix) of the beer were evaluated using the Ion pH meter (model pHB 500), AbValuer equipment and the Hanna digital refractometer (model HI96800), respectively. The beer's volatile acidity was also calculated. For this purpose, the titrimetry methodology was used using the TE-0871 distillation apparatus. In addition, the apparent attenuation (AA) of the beers was calculated according to the following formula:

$$AA = \frac{Original\ Gravity - Final\ Gravity}{Original\ Gravity}$$

3.11. High-performance liquid chromatography analysis

Sugar and ethanol content of beers after fermentation on a laboratory scale were quantified by High-Performance Liquid Chromatography (HPLC) as well as for those ones produced on a pilot scale. For this, beer samples were separated on a HPX 87H Biohad column, 300 x 7.8 mm, and kept at 45 °C and Sulfuric acid (H2SO4, 5 mmol/L) at a flow rate of 0.7 mL/min was used at mobile phase, as described by Siegfried et al. (1984). The Shimadzu LC20AT chromatograph coupled to an RID-20A refractive index detector was used.

3.12. Sensorial analysis

The beer produced in this work was evaluated, as well as the control, in regards to consumer acceptance. To this end, a sensory analysis was carried out using a hedonic scale evaluation form. Each participant received approximately 20 mL of the control and treatment samples in transparent plastic flasks and was instructed to answer the evaluation form. The participant was asked to evaluate

different attributes according to the following scores: 1 - I strongly dislike it, 2 - I dislike it, 3 - I moderately dislike it, 4 - I slightly dislike it, 5 - I don't dislike it / I don't like it, 6 - I slightly like it, 7 - I moderately like it, 8 - I like it a lot and 9 - I strongly like it. The attributes evaluated were: appearance, aroma, taste and evaluation of the product as a whole. In addition, the participant's intention to buy was also assessed using the classification: 1 - Certainly wouldn't buy, 2 - Probably wouldn't buy, 3 - Maybe wouldn't buy / Maybe would buy, 4 - Probably would buy and 5 - Certainly would buy. The beers were evaluated by 138 participants and the assays were carried out in sensory analysis cabins at the Food Technology Department (DTA/UFV) after approval by the Human Research Ethics Committee of UFV (Process CAAE 69624123.7.0000.5153).

3.13. Statistical Analysis

One-way Analysis of Variance (ANOVA), Tukey's test, Mann-Whitney test and t-test was used ($p < 0.05$).

4. RESULTS AND DISCUSSION

4.1. Yeast isolation

In this study, 27 yeast isolates were obtained. The main information about them such as codes, source, coordinates of the origin place and isolation conditions is presented in Table 4. In order to evaluate the diversity of yeasts in coffee samples, matured fruit, dry fermentation fruit and liquid fermentation samples were used for isolating step. In this study, 8 (29,6 %), 3 (11,1 %) and 16 (59,3 %) were obtained from matured fruit, dry fermentation fruit and liquid fermentation, respectively. Spontaneous fermentation of beans after the harvest can promote demucilage and improve the coffee sensorial profile (Haile & Kang, 2019). Several microorganisms are recognized as good producer of sensory compounds such as acids, alcohols, and aldehydes related to characteristics associated with caramel, chocolate and fruit notes in the product (Vilela et al., 2010; Mota et al., 2020).

Although bacteria and filamentous fungi are present, yeasts are the main microorganisms isolated from these type of fermentations (Pereira et al., 2019).

Several studies have demonstrated the microbial diversity of coffee fermentation for use as starter cultures. Mota et al. (2022) isolated *S. cerevisiae* and *T. delbrueckii* from autochthonous coffee fermentations with high fermentative efficiency, generating desirable sensory characteristics. Bressani et al. (2020) also evaluated yeasts isolated from coffee to assess the production of compounds for improving the coffee sensory profile. *Meyerozyma caribbica*, *S. cerevisiae*, *C. parapsilosis* and *T. delbrueckii* were promising yeasts for optimizing the coffee flavour. Elhalis et al. (2021) verified the potential of the *H. uvarum* and *P. kudriavzevii* to be used as starter cultures in coffee fermentations; both produced high concentrations of esters, alcohols and aldehydes, impacting positively in the coffee sensorial profile.

Table 4. Isolates obtained from mature coffee grains and samples from wet and dry coffee fermentations

Isolates	Source	Coffee Variety	Origin	Coordinates	Isolation Condition
G102 G103 G104 G107	Coffee beans	Topázio Amarelo	São Sebastião do Paraíso, State of Minas Gerais, Brazil	21º 01' 16,8''S/46º54'06,4''W	15 °C
G201 G202	Coffee beans	Colombiano	São Sebastião do Paraíso, State of Minas Gerais, Brazil	20º51'24,6'' S/ 47 º 03'09,3''W	15 °C
G204 G205	Coffee beans	Colombiano	São Sebastião do Paraíso, State of Minas Gerais, Brazil	20º51'24,6'' S/ 47 º 03'09,3''W	20 °C
G301	Coffee beans (dry fermentation)	Catuaí Vermelho	São Sebastião do Paraíso, State of Minas Gerais, Brazil	20º51'24,6'' S/ 47 º 03'09,3''W	15 °C
G306 G307	Coffee beans (dry fermentation)	Catuaí Vermelho	São Sebastião do Paraíso, State of Minas Gerais, Brazil	20º51'24,6'' S/ 47 º 03'09,3''W	20 °C

F201 F401 F901 F902 F1101	Liquid (wet fermentation)	Catuaí Vermelho	São Sebastião do Paraíso, State of Minas Gerais, Brazil	20º51’24,6’’ S/ 47 º 03’09,3’’W	20 °C
F601 F603 F605 F701 F702 F801 F803 F804 F1001 F1002 F1003	Liquid (wet fermentation)	Topázio Amarelo	São Sebastião do Paraíso, State of Minas Gerais, Brazil	20º51’24,6’’ S/ 47 º 03’09,3’’W	20 °C

Unlike the studies above mentioned, in this work yeasts isolated from coffee will be evaluated in beer fermentation processes. In this context, non-*Saccharomyces* yeasts generally related to contamination could be applied for the brewery industry, resulting in innovative beers (Iorizzo et al., 2021; Aguiar-Cervera et al., 2021). Therefore, yeasts isolated from environmental samples show great potential for biotechnological applications. Ravasio et al. (2012) isolated yeasts from fruits, soil and insects, revealing the potential of non-conventional yeasts for the optimization of beer quality. They were used in a co-fermentation system with commercial lager strains, where *W. anomalus* resulted in an increasing of fruity compounds. Hutzler et al. (2021) isolated *S. jurei* from *Fraxinus excelsior* tree and evaluated its potential for application in beer production; it was able to consume approximately 50 % of the maltotriose from wort, an unexpected finding for wild strains of this genus.

4.2. Hydrogen sulphide production

Hydrogen sulphide (H_2S) is mainly resulted from the yeast metabolism during beer fermentation. Its presence is related to the biosynthesis of sulfur-containing amino acids such as cysteine and methionine (Zhang & Cui, 2023). Both molecules exhibit a low threshold (10 mg/L), resulting in aromas commonly described as sulphurous or fetid, masking desired characteristics of the beer and negatively impacting its quality (Duan et al., 2004). In addition, H_2S can combine with other compounds in beer, providing undesirable off-flavors (Astola et al., 2023). Considering 27 isolates evaluated in this study, 3 (12.5 %) of them showed high hydrogen sulfide production (+++) (Figure 3). Therefore, these isolates were not selected for the next steps.

Besides that, 29.1 and 12.5 % showed medium (++) and low (+) H_2S production, respectively; 45.9 % of the isolates did not produce the compound (-). Considering that moderate H_2S production does not necessarily impact on beer quality, as during the biological maturation of green beer yeasts can capture it reducing the negative impacts on the beverage (Postigo et al., 2021), low and medium producers were considered able to proceed in the screening. Furthermore, to the reduction on H_2S production can be modulated by applying genetic changes; the upper expression of *NHS5* gene, for example, can suppress the formation of

hydrogen sulfide in beers produced on laboratory scale (Dequin et al., 2001). Finally, environmental factors such as wort composition can impact on the production of this off-flavor compound.

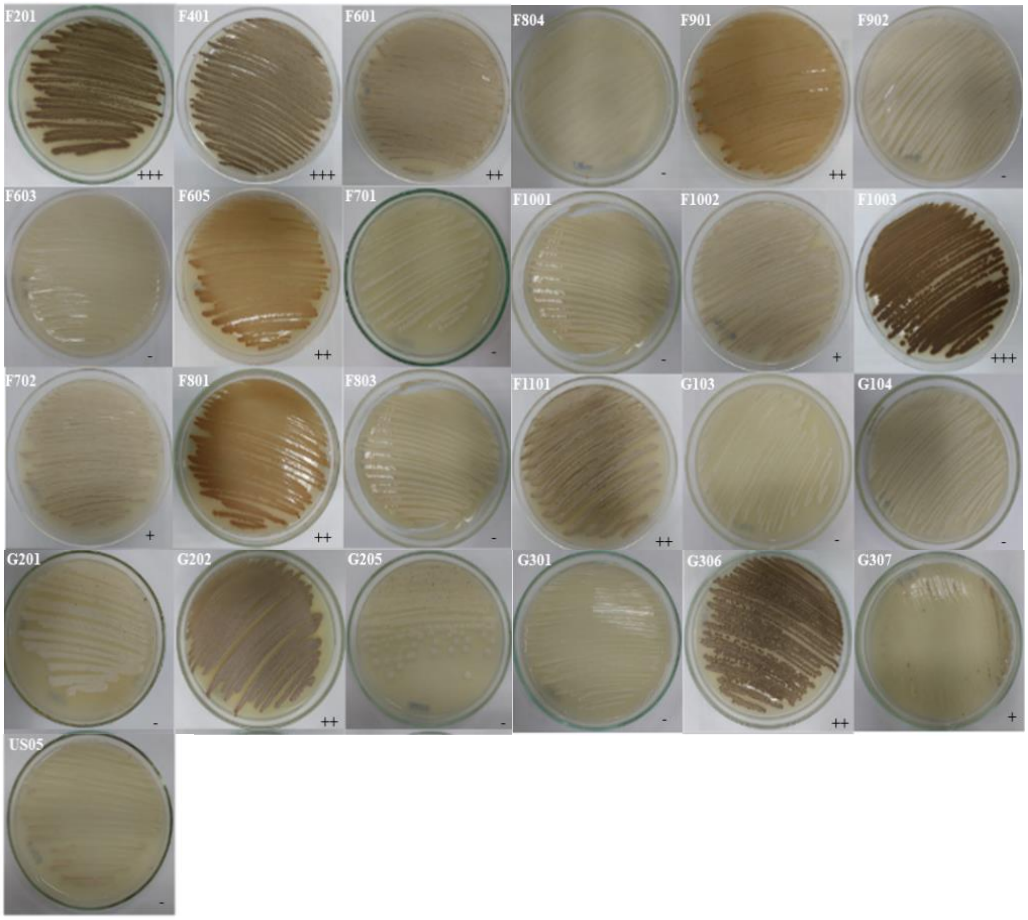


Figure 3. Evaluation of hydrogen sulfide production. The isolates were cultivated in plates containing LA medium, incubated at 28 °C for 10 days. The results were interpreted as follows: high production (+++); medium production (++); low production (+) and no production (-).

4.3. Growth capacity on different carbon sources

The isolates were evaluated for growth on media containing different sugars as the only carbon sources to verify their potential for application in beer fermentation. All isolates (100 %) were able to grow on medium containing only glucose (Figure 4). During brewing process, yeasts prefer start the fermentation from the simplest sugars present in the wort. Then, monosaccharides, such as glucose and fructose, are the first sugar consumed. Due to the abundance of maltose

in the wort, it is important know too about the yeast ability to consume this disaccharide (Álvaro, et al., 2022). Yeasts with low maltose fermentation potential can generate beer with low alcohol content and high residual sugar, compromising its quality. In general, brewer's yeasts are domesticated and present a high potential for consuming maltose, in opposite that observed for wild. However, some studies have already demonstrated non-*Saccharomyces* yeasts with this property, especially *Kazachstania, Zygotorulaspora, Kluyveromyces*, and *Torulaspora* (Toh et al., 2020).

G202, F605, F901, F702, and G104 isolates showed growth statistically equal or greater than the positive control (*S. cerevisiae* US05); this result reinforces their potential to grow in beer wort (Figure 4). The ability to metabolize maltose is related to the expression of the *MAL, AGT1* and *MTT1* genes, which encode the synthesis of membrane transporters and maltase (Álvaro et al., 2022). 52 % of the isolates evaluated did not show satisfactory growth in the medium containing maltose (F701, F1001, F603, F804, F803, G307, F601, F1101, F902 and G301). Therefore, these isolates did not proceed in the next steps. For the remaining isolates (47,4 %), the growth was statistically lower than the control; however, as they exhibited the ability to utilize sugar commonly present in beer wort, they were selected for the next steps.

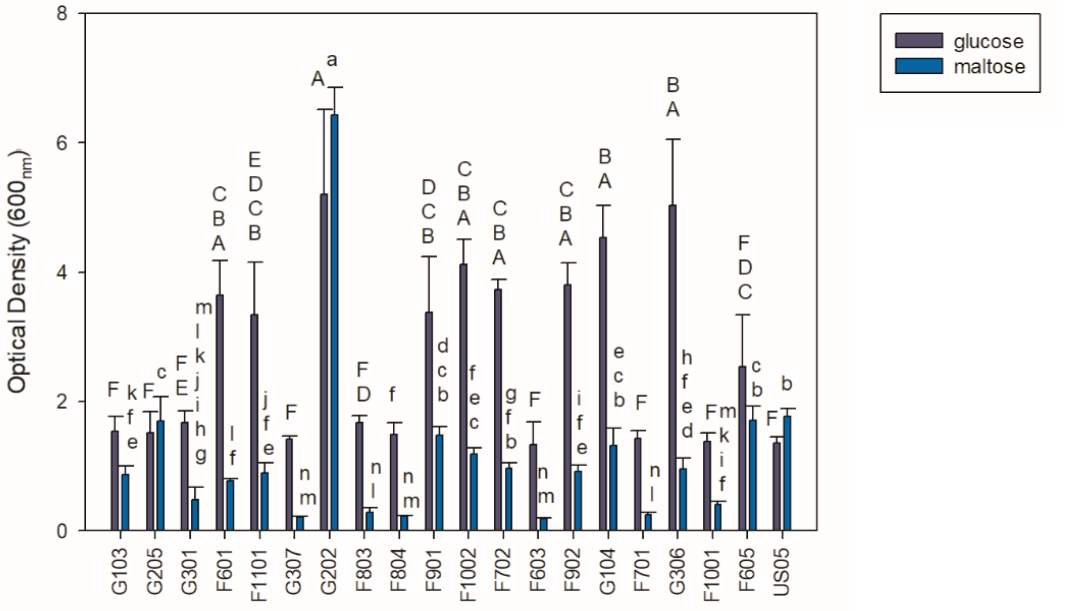


Figure 4. Growth evaluation on medium containing glucose or maltose as the only carbon source. The isolates were grown in test tubes containing YNB medium with 20 g L^{-1} of sugars for 24 hours. The test was maintained at 25 °C at 300 rpm and then the measure of OD600nm was analyzed. Different letters in the columns indicate that the treatments differed significantly at the $p < 0.05$.

After maltose consumption, yeasts utilize trisaccharide, such as maltotriose. For this reason, the isolates were also evaluated for their ability to grow on culture medium containing maltotriose as the only carbon source. It is a complex sugar generally less used by the yeast (Iorizzo et al. 2021). Strains conventionally applied in brewing process can utilize maltotriose as carbon source, which is important since it is the second most abundant sugar in the brewing wort (Magalhães et al., 2016). The rate of utilization varies depending on the strain, being its consumption directly related to process known as beer attenuation. The more maltotriose is consumed, the greater is the attenuation of the beverage. Maltotriose input depends on the maltose transporters or specific systems; however, the transport mechanisms are not well understood at this moment.

Although growth capacity lower than the observed for US05 yeast, the isolates G202, F901, F702, G205, F601 and F605 were able to growth in the substrate with only maltotriose as carbon source (Figure 5). It demonstrates their ability to transport maltotriose into cytoplasm and metabolize it. In contrast, was not observed growth for F1101, F902, F1002, G103, G306 and G104. The

incapacity to use the second most abundant sugar in beer wort may reflect on the sensory profile of the beverage as well as reduced its microbiological stability. Thereat, these isolates were excluded.

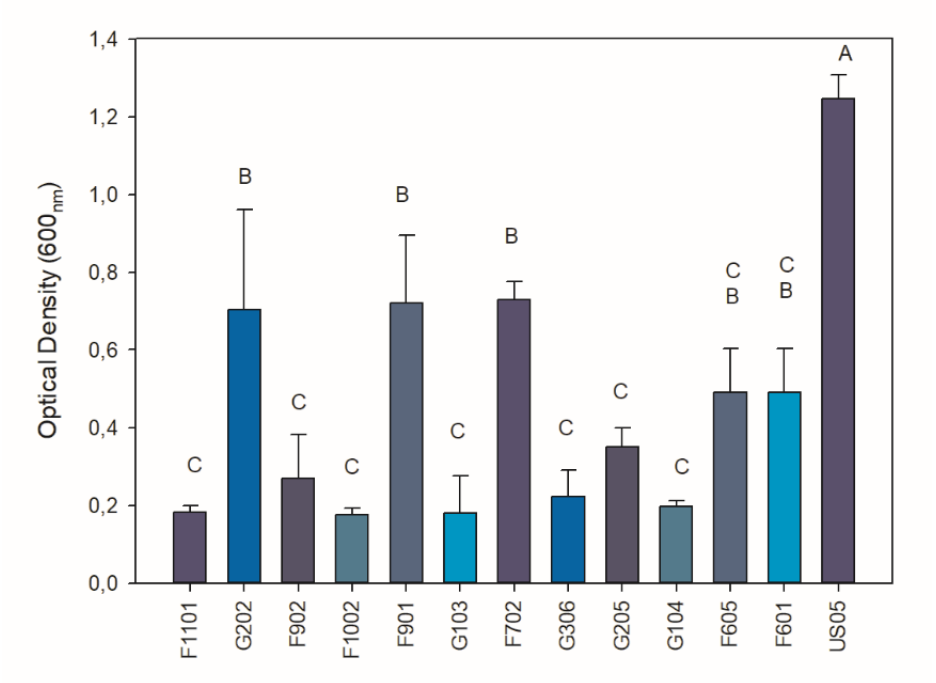


Figure 5. Evaluation of growth on medium containing maltotriose as the unique carbon source. The cultures were inoculated in YNB medium with 20 g L^{-1} of maltotriose. Assays were conducted in microplates and growth evaluation was conducted after 24 hours. Different letters in the columns indicate that the treatments differed significantly at the $p < 0.05$.

4.4. Phenolics compounds production

Conventional brewer's yeasts are classified as POF^+ or POF^- according to their ability to produce phenolic compounds. In the beer wort, phenolic precursors such as *p*-coumaric and ferulic acids are present and can undergo oxidative decarboxylation, generating phenolic flavors for the beer. For this, yeasts must present genes for coding phenyl acrylic acid decarboxylase and ferulic acid decarboxylase (Gallone et al., 2016). Therefore, strains able to synthesize these proteins are classified as phenolic positive (POF^+). The most part of conventional yeasts are POF^- as they suffered silencing and nonsense mutations in that gene (Mukai et al., 2014).

Ferulic acid, a constituent of beer wort, is a precursor for the formation of volatile phenolic compounds (4-vinylguaicol). When it is converted by enzymatic

activity from yeast metabolism, compounds related to clove aroma in beer are produced. Yeasts able to generate 4-vinylguaicol are commonly applied in Weizen production, a popular German beer (Astola et al., 2023). Most wild yeasts can produce high concentrations of phenolic compounds. On the other hand, brewer's yeasts do not show this ability due to domestication process. Therefore, the isolates were grown on medium containing this precursor to evaluate the production of phenolic compounds of interest. F605, F901, and G202 isolates were positive for clove aroma production (Table 5). Thus, these isolates could be employed in certain beer styles production such as Belgian and German wheat beers, since clove aromas are required for them. Interestingly, F601, F702 and G205 isolates were not able to release 4-vinylguaicol, indicating their possibility to be applied in the production of most beer styles.

Table 5. Evaluation of the production of 4 vinyl-guaiacol

Isolates	Phenolic Compound Production
F601	Negative
F605	Positive
F702	Negative
F901	Positive
G0202	Positive
G205	Negative

4.5. Tolerance to stress factors inherent to brewing

Several stress conditions are observed during beer production, impairing yeasts and the fermentation process. Assays to evaluate the tolerance to stress factors can provide important insights for selecting the yeasts more appropriated for specific beer styles. For this, isolates were grown at different conditions, such as alcohol concentration, pH values and incubation temperatures.

All isolates, except G202, were able to grow at pH 4.5 (Figure 6). During the wort fermentation, pH is decrease; it can potentially cause damage to the microorganisms. These results indicate that these isolates could be applied in co-fermentations with yeasts commonly used in brewing process, for example. F901 and F605 showed growth at pH 3.5; these isolates could be used for acid beer production, which pH values range from 3.3 to 3.5. Considering sour beers are

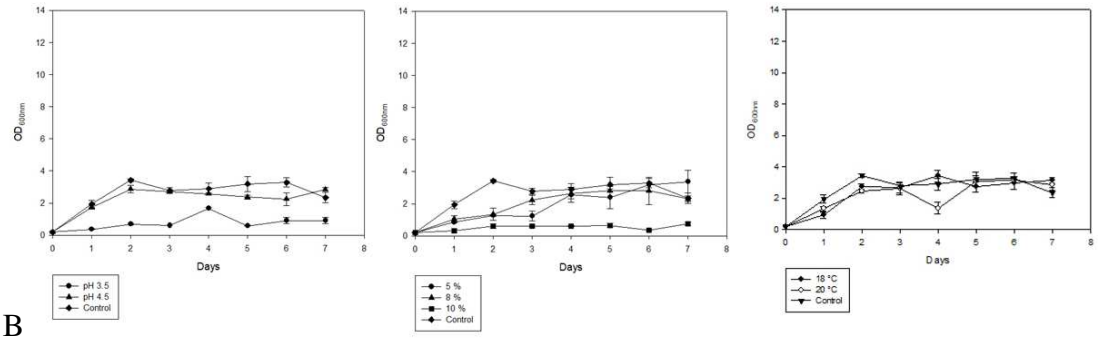
produced acidifying the wort before the inoculation the yeasts must be able to tolerate low pH values.

Brewer's yeasts are also expected to tolerate the presence of alcohol, which can affect the cell wall integrity, as well as the cytoplasmic membrane fluidity and functioning. In addition, harmful effects on proteins structure and cellular activity can be observed (Foster et al., 2022). In this context, alcohol-tolerant yeasts are indispensable for brewing processes to guarantee beer quality. Considering the modernization of beer industry in last years, an increasing demand for yeasts more tolerant to high alcohol content has been observed in order to allow the yeast recovery after the fermentation and for production of high-density beers. It has driven novel studies in the field, specially about prospecting new yeasts or obtain strains of interest through genetic engineering tools. For example, Yang et al. (2023) carried out adaptive laboratory evolution trials to obtain brewing strains with greater alcohol tolerance, getting yeast strains with tolerance to high alcohol concentrations (12 % v/v).

In this work, the isolates were grown in media containing 5, 8 or 10 % ethanol (v/v). F901 and F605 isolates were able to grow under 10 % alcohol, indicating their potential to be used for high density beer production (Figure 6). Alcohol tolerance is related to polygenic factors, depending on several metabolic pathways. It has been reported that the potential of trehalose accumulation inside the microbial cell can increase the alcohol resistance (Yang et al., 2023). It's important to highlight that in this study the isolates were evaluated under optimal growth conditions, which may not accurately reflect trustworthy beer production conditions. G202 and G205 isolates (Figures 6) did not grow at the alcohol levels evaluated; then, they were considered as not promisors' yeasts for brewing. In traditional methods for low-alcohol beer production, the beer sensory profile can be impaired due to the alcohol reduction as it is removed relevant compounds for the flavor. In this sense, non-*Saccharomyces* yeasts with low alcohol production have been evaluated (Karaoglan et al., 2022). However, in this work non-tolerant isolates were eliminated from the screening.

Regarding the temperature, all isolates showed growth in the main conditions established for most beer styles. Ale beers are produced at temperatures ranging from 16 to 24 °C. In view of the results obtained until this step, F605 and F702 isolates were selected for taxonomic identification.

A


OD600nm
Days
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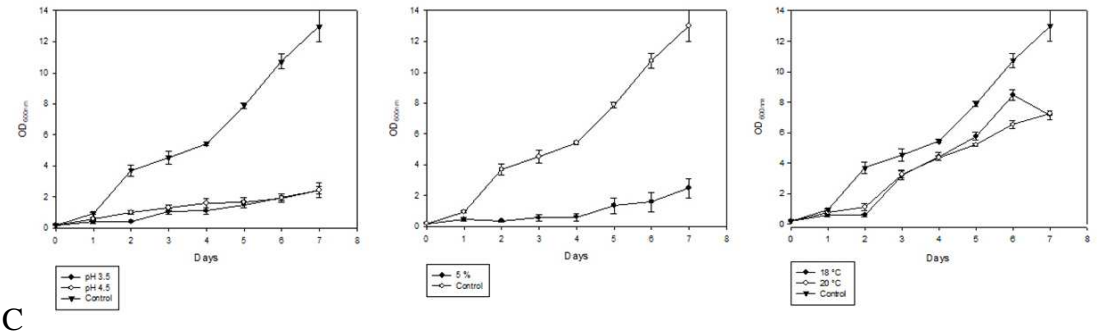
pH 4.5
Control
5 %
8 %
10 %
Control
18 °C
20 °C
Control

B


OD600nm
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pH 3.5
pH 4.5
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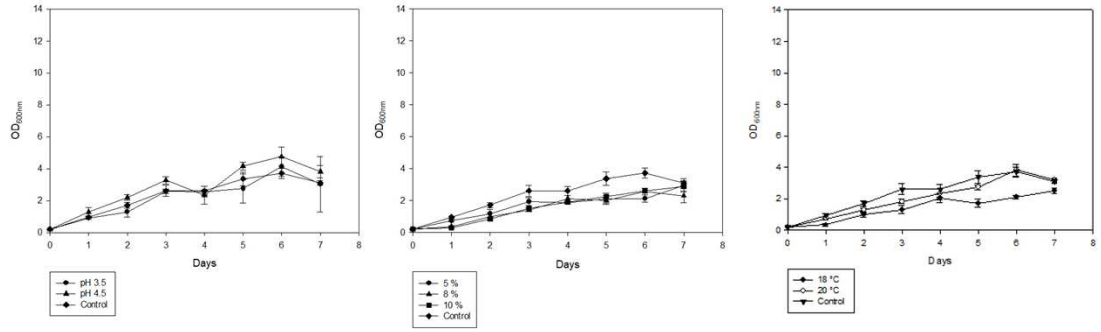
Control
18 °C
20 °C
Control

C


OD600nm
Days
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pH 4.5
Control
5 %
8 %
10 %
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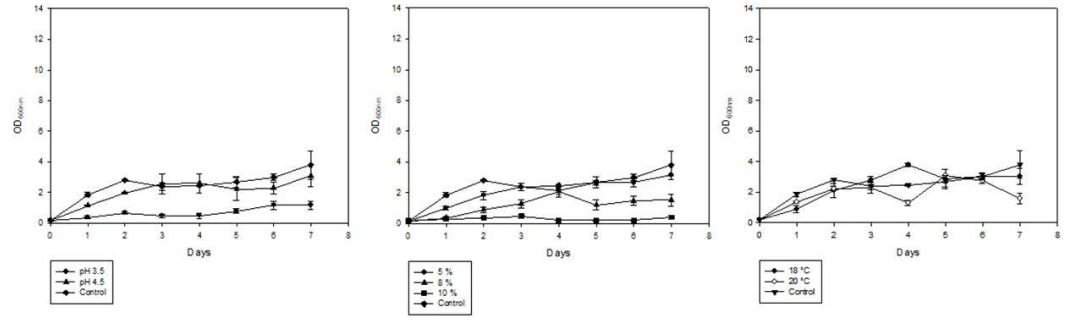
18 °C
20 °C
Control

D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20 °C
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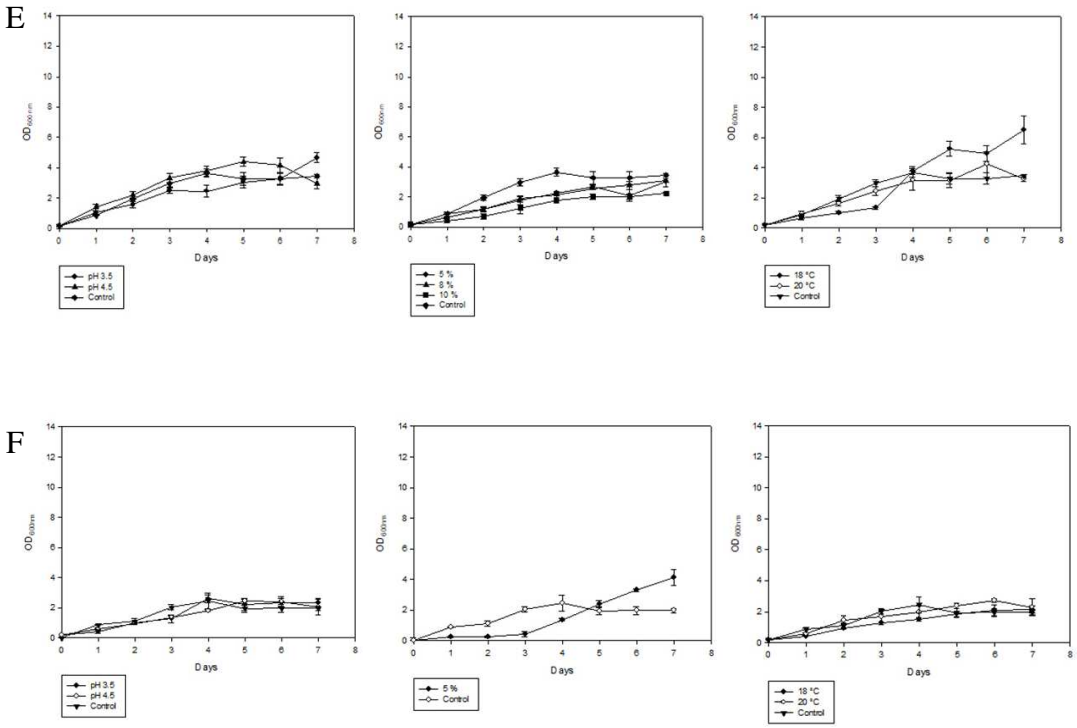

Figure 6. Evaluation of the growth of isolate under stress conditions. The cultures were grown in YPD medium with the respective stress factors (alcohol, pH change and temperature variation) at 25 °C for 7 days and then the OD600 nm measurement was determined every 24 hours. The control for all the tests was conducted in YPD and incubated at 25 °C (A: F601, B: G202, C: F901, D: F702, E: F605 e F: G205).

4.6. Taxonomic identification

Non-conventional yeasts can produce a wide variety of compounds in comparison with *Saccharomyces* spp., contributing for the production of novel beer profiles. Prospecting innovative yeasts is complex and involves the study of parameters such as tolerance to stress conditions, sugar consumption and production of metabolites of interest. Considering the results obtained in this study, two isolates were selected and submitted to taxonomic identification. F605 was identified as *W. anomalus*, and F702 as *T. delbrueckii,* according to Table 6.

Table 6. Identification of selected isolates

Isolate	Identification	Similarity Index (%)	Coverage (%)
F605	*Wickerhamomyces anomalus*	99,9	98.2
F702	*Torulaspora delbrueckii*	99,4	95.3

All isolates evaluated were obtained from Brazilian coffee beans, a source with great microbial diversity depending on the place of origin. They can impact the quality of the beverage due to metabolites produces during coffee fermentation, a step that can be applied on coffee beans after the harvest (Prakash et al., 2021). It is worth reinforce that the microorganisms prospected here came from Alta Mogiana region, a place in Minas Gerais State known worldwide for its coffee quality.

Studies about yeast diversity in coffee beans have revealed several majority species, including *Torulaspora* and *Wickerhamomyces* (Elhalis & Zhao, 2022). *T. delbrueckii* is the most studied non-conventional yeast for wine production (Benito et al., 2018); its application for other fermented beverages production has been proposed (Fernandes et al., 2021). *T. delbrueckii* strains can play an important role on sensorial profile of the product due to the production of compounds such as esters (Liu et al., 2021). In addition, they are able to transform compounds present in hop, resulting in more complex and particular flavour (Michel et al., 2016). Finally, *T. delbrueckii* is recognized to produce low amounts of undesirable compounds, for example acetaldehyde, acetoin and acetic acid (Canonico et al., 2016).

In turn, *W. anomalus* has also been evaluated for beer production, although it has been commonly reported as an eventually contaminant (Aponte et al., 2022). Pinto et al. (2022) highlighted the potential of *W. anomalus* to produce interesting compounds for brewing. Postigo et al. (2022) demonstrated the viability of *W. anomalus* strains to be employed for production of beers with low alcohol content or in co-fermentations with conventional brewer's yeasts, improving the beer quality. Pinto et al. (2023) isolated and characterized a *W. anomalus* strain,

demonstrating its role on production of complex sensory compounds, such as esters and phenolic compounds.

In this context, both isolates were selected for fermentation characterization on a laboratory scale in order to evaluate sugar consumption and ethanol production.

4.7. Characterization of the fermentation profile on a laboratory scale

Isolates F605 and F702 were selected for fermentation trials on a laboratory scale, where the consumption of the main sugars in the wort (glucose, maltose and maltotriose) and the production of ethanol were evaluated. With this information, it is possible to define the fermentation potential of the isolates and direct the production process, validating whether the isolates are capable of fermenting the wort individually or whether they should be used in co-fermentations.

The characterization of sugar consumption showed that the isolates had a low fermentation potential. It is expected that environmental yeasts take longer to ferment and exhibit slow consumption compared to commercial yeasts, which are domesticated and adapted to brewing wort. Postigo et al. (2022), for example, had already reported the same situation in an evaluation conducted with isolates of the species *Saccharomyces cerevisiae, Hanseniaspora guilliermondii, Metschnikowia pulcherrima, T. delbrueckii and ZygoSaccharomyces bailii,* where all of them were unable to produce alcohol and utilize the maltose present in the wort and, for this reason, beer production was conducted in co-fermentation.

It is important to note that the isolates evaluated grew in a medium containing maltose or maltotriose as the only carbon sources in the screening tests. However, the results obtained with chromatographic characterization were different. This may be related to the complexity of the medium and fermentation conditions, such as pitching rate.

The F702 isolate had higher glucose consumption compared to the F605 yeast, but took a day longer to consume all the sugar in the wort compared to the control. The F605 yeast did not consume all the glucose in the wort, even after seven days of evaluation, which indicates low growth and reduced fermentation rates (Figure 7).

Regarding the consumption of maltose and maltotriose, both isolates evaluated were unable to metabolize them during the seven days of the experiment (Figure 8). This reflects the need to use them in co-fermentations with a yeast with attenuation potential. It is also worth noting that various factors can influence the utilization of sugars, such as fermentation temperature, type of fermenter, nutrient composition and aeration. In this sense, the results obtained in laboratory-scale fermentation trials provide input for characterizing the isolates, but it is necessary to characterize the yeasts in a real production environment.

It should also be noted that even on a production scale, different production conditions need to be studied and tested in order to achieve the best results. Non-conventional yeasts can exhibit low fermentation efficiency and, for this reason, the fermentation and maturation time needs to be longer than in conventional fermentations. In this sense, it is necessary to establish the best process conditions.

Also in this assay, ethanol production was evaluated, which showed the low potential of the two isolates to produce alcoholic beers (Figure 9). Because of this property, yeasts of the *T. delbrueckii* species have been studied and proposed for the production of low-alcohol beers. Nikulin et al. (20220), for example, evaluated different yeast species and concluded that a strain of *T. delbrueckii* was the most promising. The isolate had a sensory profile similar to the control and was able to produce beers with 0 % alcohol or very close to it. In another study, Drosou et al. (2022) evaluated two different strains of *T. delbrueckii* and verified the production of 3.90 and 5.50 % alcohol by volume (ABV), demonstrating a lower content when compared to the control (4.80 - 5.80 % ABV). Isolates belonging to the *W. anomalus* species have also been proposed for the production of beers with low alcohol content, as they do not produce low alcohol concentrations and exhibit a promising sensory profile (Capece et al., 2018; Simões et al., 2023).

These yeasts can also be used in alcoholic beers, but they need to be associated with a yeast capable of adequately attenuating the wort. Several studies have demonstrated the potential of these species in co-fermentation to optimize the sensory profile and generate innovative beers (Canonico, et al., 2018; Capece et al., 2018). In this study, isolate F605 was selected due to the best sensory profile observed, and was used to produce beer in co-fermentation with a conventional yeast, with the aim of evaluating the beer on a pilot scale.

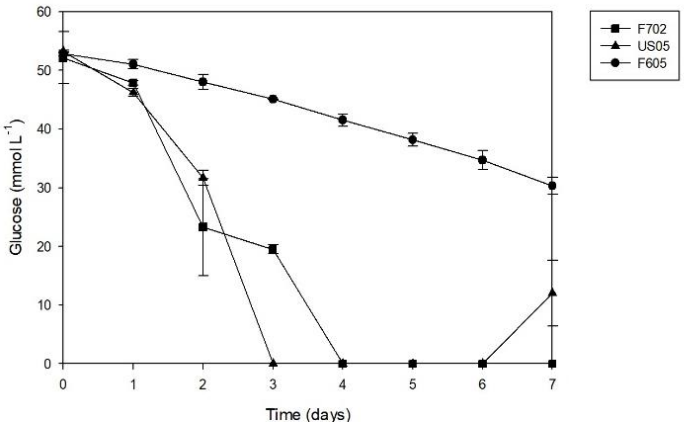

Figure 7. Characterization of glucose consumption by HPLC analysis. The yeasts were subjected to a laboratory-scale fermentation process by cultivating them in 500 ml of wort for seven days at 20 °C. Daily samples were collected to evaluate glucose consumption.

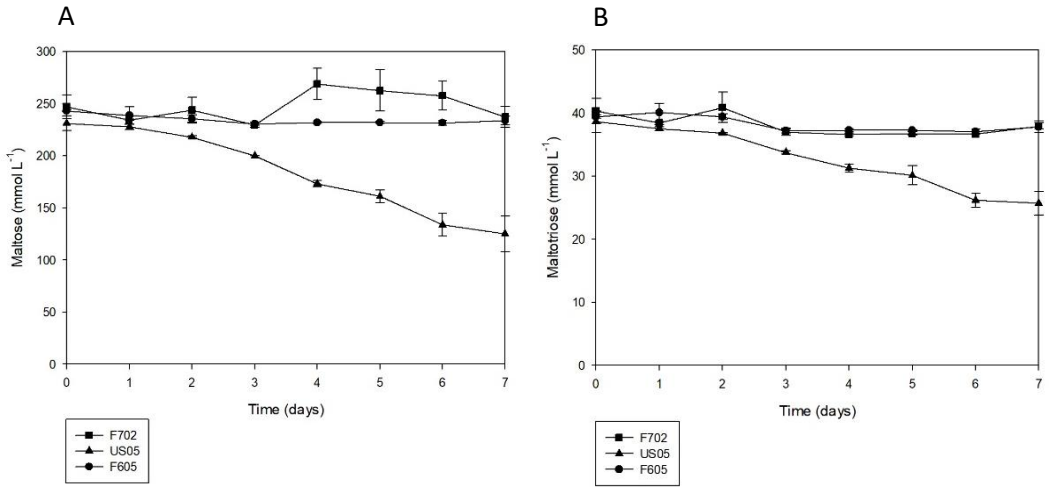

Figure 8. Characterization of maltose (A) and maltotriose (B) consumption by HPLC analysis. The yeasts were subjected to a laboratory-scale fermentation process by cultivating them in 500 ml of wort for seven days at 20 °C. Daily samples were collected to evaluate maltose and maltotriose consumption.

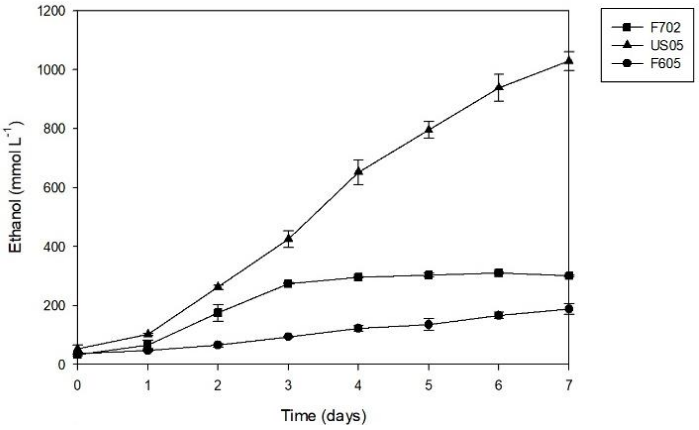

Figure 9. Characterization of ethanol production by HPLC analysis. The yeasts were subjected to a laboratory-scale fermentation process by cultivating them in 500 ml of wort for seven days at 20 °C. Daily samples were collected to evaluate ethanol production.

4.8. Characterization of the fermentation profile on a pilot scale production

The beers obtained were characterized in relation to alcohol content, sugar density and pH (Figure 10). The results indicated 5.2 and 5.6 % alcohol in the control and treatment, respectively. This showed that the conventional yeast used in the co-fermentation was able to ferment and attenuate the beverage. In addition, isolate F605 was able to tolerate the alcohol content, according to the expected since its capacity to growth at 10 % alcohol in screening step. There are studies identifying strains of this species with the ability to tolerate up to 12.5 % alcohol and, for this reason, these yeasts have been widely studied for application in several biotechnological processes (Padilla et al., 2018).

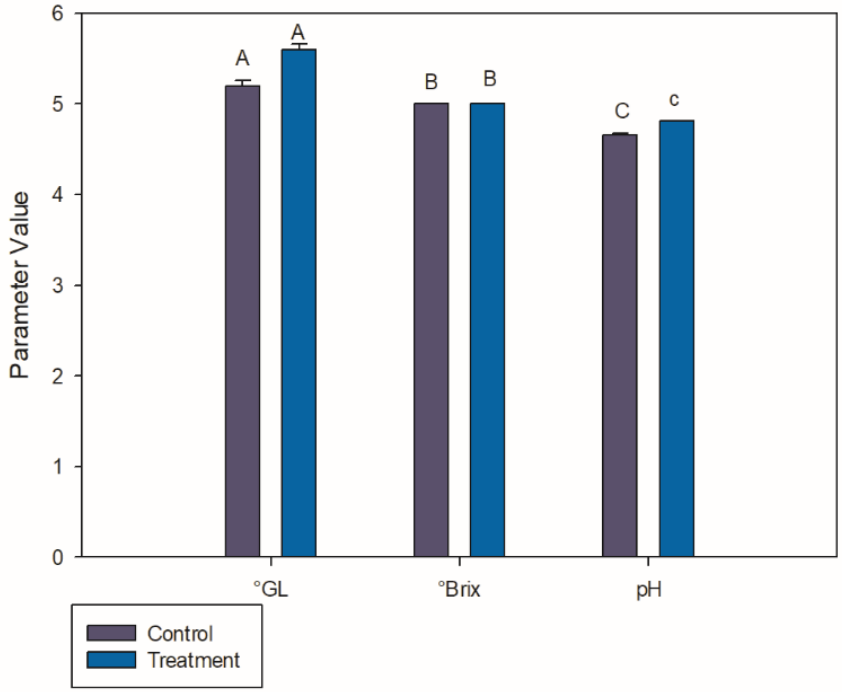

Figure 10. Evaluation of the beers produced in relation to alcohol content (° GL), soluble solids content (° Brix) and pH. In order to characterize the beverage, a pilot batch of beer was produced using the selected yeast (F605) in a co-fermentation process with the conventional US-05 yeast. To control the experiment, a fermentation was conducted with a pure culture of US-05. After the brewing, samples were collected to assess the alcohol content, soluble solids and pH measurement. The indication above each column represents the statistical analysis carried out for each parameter evaluated, where different letters indicate that the treatments differed significantly at the $p < 0.05$.

The alcohol content resulting from co-fermentations between *S. cerevisiae* and *W. anomalus* can be lower in comparison to fermentation using only the conventional yeast. However, these is a strain-dependent effect (Liu et al., 2021). In this study, the values obtained for control and treatment were not significative different, demonstrating that the F605 isolate did not affect the alcohol content of the beer ($p < 0.05$).

Before inoculating the yeast, the worth pH and SSC were measured (Table 7). SSC values for both were also statistically similar, indicating that attenuation was not impaired by the presence of a non-*Saccharomyces* strain. Furthermore, the beer produced with isolate F506 showed 85 % of apparent attenuation. This value is considered high and indicates that approximately the same proportion of sugars

were converted into alcohol and carbon dioxide. The conventional yeast US-05 (Fermentis®) showed apparent attenuation ranging from 78 to 82 %, according to manufacturer, which corroborates the data obtained in this study, indicating that fermentation was completely finished.

Table 7. Worth pH, SSC and apparent attenuation of the beers

	pH		SSC (°Brix)		Apparent attenuation
	Wort	**Beer**	**Wort**	**Beer**	
Control	5.58 ± 0.01	4.66 ± 0.015	10.5 ± 0.01	5.0 ± 0.00	85 %
Treatment	5.58 ± 0.01	4.81 ± 0.005	10.5 ± 0.01	5.0 ± 0.00	85 %

Although *W. anomalus* has low biomass production and slow growth, it can compete with *S. cerevisiae* and harm wort fermentation (Padilla et al., 2018). In this work, the results observed for beer attenuation and alcohol content indicate that the fermentation process occurred satisfactorily and that both strains used were able to growth in the beer wort. However, adjustments aimed at optimizing the fermentation process in order to establish the best ratio of each yeast used are necessary.

Regarding pH values, a significant difference was observed between the evaluated treatments, with higher values for beer produced with the yeast mixture. Organic acids generated by yeast metabolism correspond to the main cause of reducing the pH, which can impact the sensorial quality. In any case, the pH values for both treatments were within the expected limits for this type of beer (3.8 – 4.7).

The organic acids production was also evaluated, as shown in Figures 11 and 12. Adequate concentrations of organic acids are important not only for pH, but also for flavor, foam stability and microbial susceptibility (Araujo et al., 2002; Li et al., 2007). These compounds can be generated during the Krebs cycle and its concentration depends on the yeast-type and the fermentation conditions (Willaert, 2012). The volatile acidity of the beer was another parameter assessed to demonstrate the presence of acids in the beverage. The control beer and the treatment showed 0.066 and 0.063 volatile acidity (g acetic acid/100 ml of beer),

respectively, demonstrating that both beverages are in the range of values normally found in beer (0.057 to 0.145 g /100 mL of beer).

W. anomalus strains are known for their high production of acetic acid, which can be a sensory problem for beer; however, it depends on the strain used for brewing. The beer produced with isolate F605 showed higher production of acetic acid in comparison to the control; meantime, the concentration observed (3.084 mmol/L (180 mg/L)) is expected for most beer styles, as it is below the perception threshold (around 200 mg/L) (Bouchez & Vuyst, 2022).

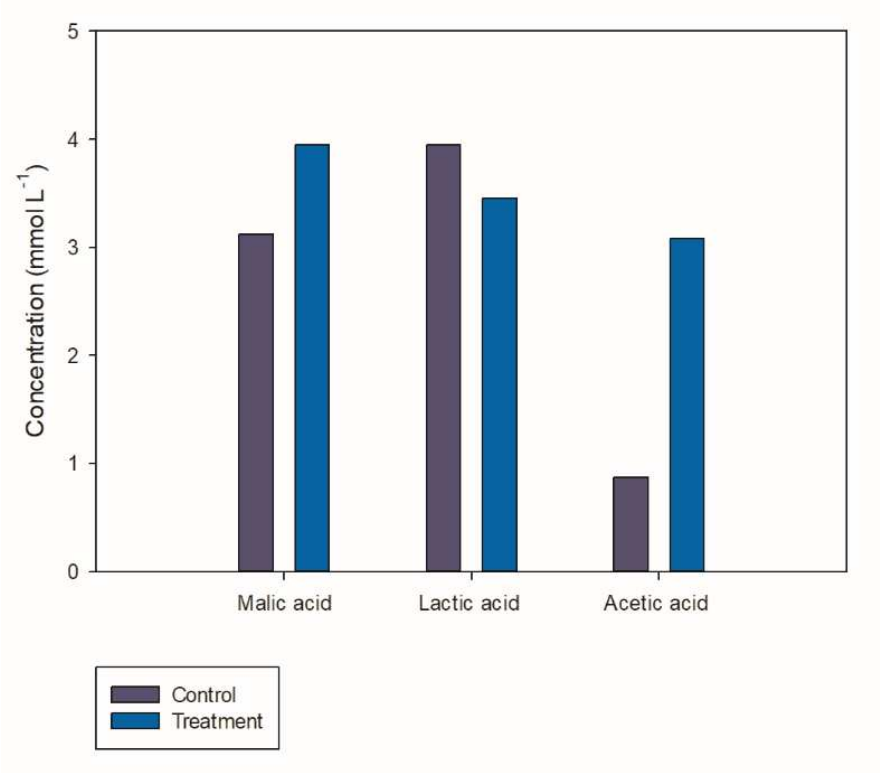

Figure 11. Organic acids concentration in beers by HPLC analysis. Samples were collected after the production of the pilot batch of beer to characterize the organic acid profile. The treatment refers to the batch produced with the F605 isolate in co-fermentation with the conventional yeast US-05 and the control refers to the production using only the pure culture of US-05.

Lactic and malic acids were also quantified. Excess malic acid can impair the beer drinkability, while lactic acid can provide off-flavors (Tyrell & Fischer, 2014). Their concentrations depend on the strain, yeast viability and the production process. The beer produced had 3.452 mmol/L (310 mg/L) of lactic acid, which indicates normality, as it is below the perception threshold (400 mg/L) (Witrick et

al., 2017). Regarding to malic acid, the concentration normally observed for beers range from 100 to 350 mg/L, depending on beer style and production conditions (Walker et al., 2012). In this study, both (control and treatment) showed higher values (3.120 mmol/L (418 mg/L) and 3.949 mmol/L (529 mg/L)), which may indicate some failures during the brewing process.

4.9. Sensorial Analysis

To evaluate consumer preference and acceptance, a sensory analysis was carried out, evaluating parameters such as the final product, appearance, taste and aroma. In relation to the global evaluation, both treatments received ratings of around 6, which indicates that the participants liked the beverage slightly. It was also possible to observe that the participants did not note any differences between the evaluation of the control and the treatment (Figure 13). It reinforces that the use of isolate F605 in co-fermentation could be a strategy to satisfy the great consumer demand for new products since it was as well evaluated as the beer using conventional yeast.

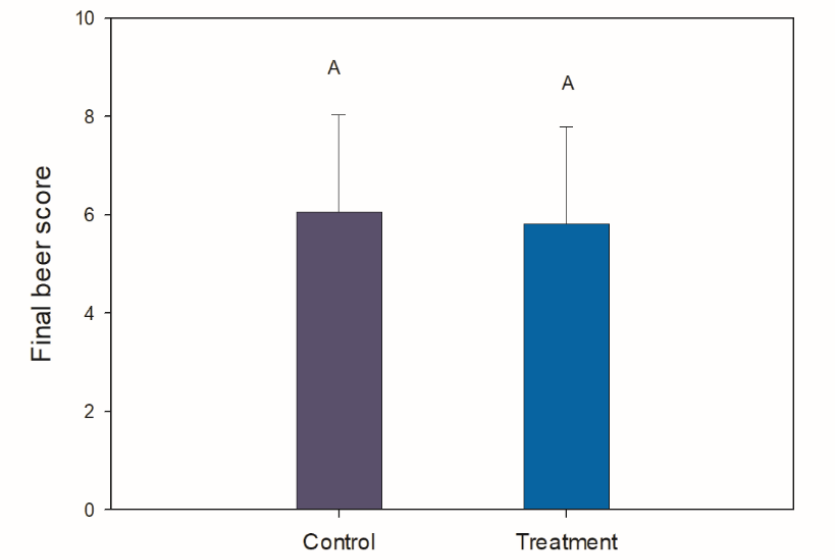

Figure 12. Classification of the beers produced in relation to the global evaluation. The beers produced were evaluated in terms of consumer preference and acceptance. To do this, the participants evaluated the final score of the beer produced from the co-fermentation between isolate F605 and US-05, as well as the control of the experiment (fermentation using only the conventional yeast US-05). Different capital letters indicate that the treatments differed significantly at the $p < 0.05$.

Several studies have proposed the use of *W. anomalus* strains to perform in co-fermentation with *S. cerevisiae*, but most are related to wine production. Wang et al. (2023) evaluated different strains of *W. anomalus* in co-fermentation with traditional yeasts and demonstrated improvements in wine sensorial profile such as intensity, astringency, complexity and flavour persistence. In addition, the strains evaluated were able to provide higher concentrations of esters, total phenolics and higher alcohols. Cañas et al. (2014) also evaluated the effect of *W. anomalus* on the production of red wines, showing an improvement of sensory complexity from the optimization of acetate and ethyl ester production. The sensory analysis carried out showed that the wine produced with *W. anomalus* was preferred by 71.5 % of the tasters.

Despite the evidences mentioned above, there are still few studies evaluating co-fermentation of beer including *W. anomalus* and *S. cerevisiae*; however, there is great potential due to the sensory potential provided by this yeast. Osburn et al. (2018) evaluated the potential of a novel *W. anomalus* strain in replacement of lactic acid bacteria for sour beer production; demonstrating its ability to produce significative contents of lactic acid and ethanol. The authors obtained a clean, aromatic and fruity beverage, with notes of pear, apple and apricot.

The F605 isolate evaluated in this work also has potential for use in beer production, mainly due to the properties exhibited in the screening tests. However, higher scores could be obtained after adjustments in yeast populations and production process. These adjustments are indispensable for avoiding competition between the yeasts trough co-fermentation and optimizing beer sensory profile. Canonico et al (2018) evaluated the potential of *W. anomalus* to be used in co-fermentation with *S. cerevisiae* for brewing considering different ratio (1:1, 1:10 and 1:20, *S. cerevisiae* : *W. anomalus*). For all ratio evaluated, the wort was completely attenuated, except for 1:20 ratio, where *W. anomalus* dominated the fermentation process.

The beers were also evaluated for appearance, aroma and taste. Control and treatment's average scores for appearance were 6.8 and 6.77, respectively. Regarding to aroma, the average scores for both were 6.4 and 6.2. No significant differences were identified between the treatment and the control ($p < 0.05$) for appearance and aroma (Figure 14). Taste score of control was higher (5.78) than treatment (5.40); in this sense, the taster panel classified it ranging from "neither

liked nor disliked" to "slightly liked". Therefore, this result suggests that further adjustments in the brewing conditions proposed in this study are required in order to improve sensorial quality of the beer.

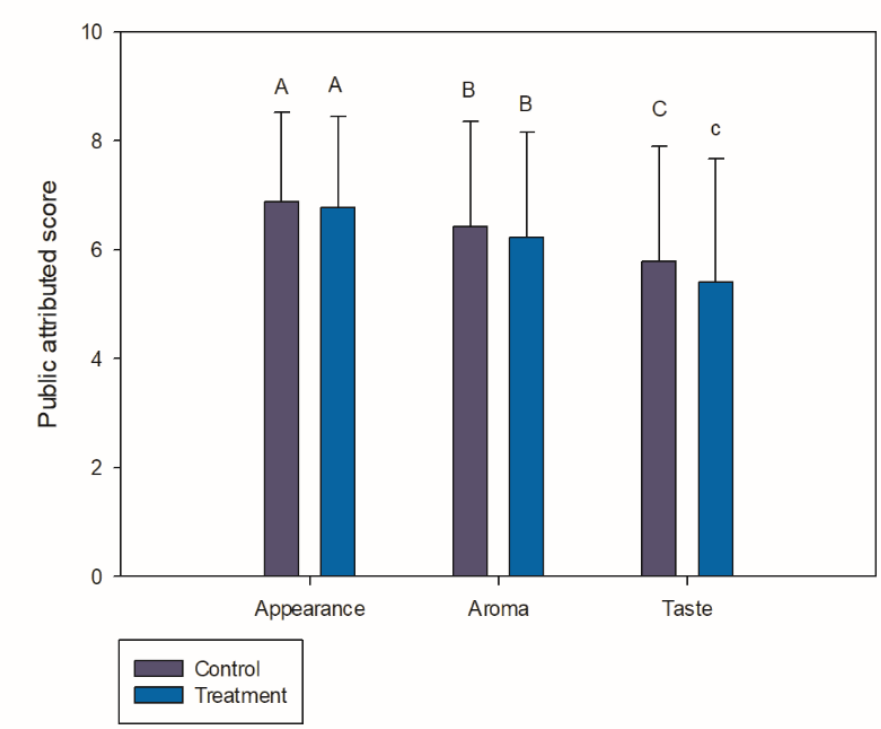

Figure 12. Classification of the beers produced in relation to the appearance, aroma and taste. The beers produced were evaluated in terms of consumer preference and acceptance and for this purpose the parameters appearance, aroma and taste were evaluated. The indication above each column represents the statistical analysis carried out for each parameter evaluated, where different letters indicate that the treatments differed significantly at the $p < 0.05$.

Finally, it is worth highlighting that few studies have evaluated the use of *W. anomalus* for production of fermented alcoholic beverages; therefore, more studies are necessary to characterize this yeast in co-fermentation models with conventional yeasts. The evaluation of ideal ratio, fermentation conditions and the influence of raw materials used in beer production are parameters to be used in future studies.

5. CONCLUSIONS

In this study, yeasts were isolated from Brazilian coffee beans and environment with great microbial diversity for brewing application. A new yeast isolate (F605) identified as *W. anomalus* showed significative tolerance to stress factors commonly observed during beer production and low production of compounds such as hydrogen sulphide. In addition, it was able to grow in co-fermentation with a conventional yeast (*S. cerevisiae*). The beer produced by them presented alcohol content and pH values expected, indicating that the conventional yeast was capable of fermenting the beer wort even in the presence of *W. anomalus* isolate. The beer showed adequate concentrations of organic acids and was classified positively by the taster panel. Further studies are needed to evaluate different yeast ratio during co-fermentation, as well as fermentation conditions, in order to optimize the beverage produced and improve its quality.

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