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Review

Maltose-Negative Yeast in Non-Alcoholic and Low-Alcoholic Beer Production

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Abstract: Although beer is a widely used beverage in many cultures, there is a need for a new drinking alternative in the face of rising issues such as health concerns or weight problems. However, non-alcoholic and low-alcoholic beers (NABLAB) still have some sensory problems that have not been fully remedied today, such as "wort-like"/"potato-like" flavours or a lack of aroma. These defects are due to the lack of alcohol (and the lack of the aldehyde-reducing effect of alcohol fermentation), as well as production techniques. The use of new yeast strains that cannot ferment maltose—the foremost sugar in the wort—is highly promising to produce a more palatable and sustainable NABLAB product because production with these yeast strains can be performed with standard brewery equipment. In the scientific literature, it is clear that interest in the production of NABLAB has increased recently, and experiments have been carried out with maltose-negative yeast strains isolated from many different environments. This study describes maltose-negative yeasts and their aromatic potential for the production of NABLAB by comprehensively examining recent academic studies.

Keywords: maltose-negative yeast; brewing; non-alcoholic beer; low-alcoholic beer; aroma; NABLAB

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1. Introduction

Beer is a nutritious fermented beverage that has been widely consumed since ancient times [1]. In the last few decades, the growing awareness by consumers about health and well-being, as well as discussions about damage caused by alcohol consumption, have increased consumers' preferences for non-alcoholic beverages [2,3].

In the EU, "low-alcohol beers", with alcohol content from 0.5% v/v to 1.2% alcohol v/v, are separated from non-alcoholic beers. In the United States, the upper limit for "non-alcoholic beer" is 0.5% alcohol v/v. In countries with religious proscriptions, an alcohol content of 0.05% v/v should not be exceeded in beverages [3,4].

Since alcohol makes an essential contribution to the flavour of beer, for most consumers, non-alcoholic beers lack flavour and this can be easily perceived. Additionally, potato-like flavours develop in the de-alcoholising process when thermal processes are employed. Until recently, *non-Saccharomyces* yeasts were considered undesirable in beer/wine making. However, in more recent studies, these unconventional yeast strains have been shown to create fruity aromas while still producing little or no alcohol [5,6]. As is documented, non-*Saccharomyces* yeast strains are commonly sensitive to ethanol stress while also provide a distinctive aroma and taste [7]. Recent studies on NABLAB indicate that using non-*Saccharomyces* yeast strains in the fermentation process—instead of the more traditional de-alcoholising process—can effectively get rid of sensory faults, offer new non-traditional flavours (without additional investments in specialised equipment), and reduce the carbon footprint of brewing [5]. These special yeasts cannot ferment maltose due to deficiencies

in the maltose transporter and maltase enzyme, and are referred to as being "maltose-negative" [8–10]. Brewers and researchers are working to identify or develop alternative maltose-negative yeasts from various fermented products and non-brewing-related environments. These maltose-negative yeasts can have an improved aroma potential but their safety in a food context is paramount [11]. Generally, isolating yeasts from fermented food systems can be safer than non-food-source yeasts [6]. There are several short "safe lists" by the European Food Safety Authority (EFSA) Biohazard Panel (Qualified Presumption of Safety—QPS), by the United States Food and Drug Administration (FDA) (Generally Recognized as Safe—GRAS), and by some other organisations (such as the International Dairy Federation—IDF/European Food and Feed Cultures Association—EFFCA) regarding this new field of study.

Many studies are still very recent, and more research is needed on flavour properties and safety. This review summarises recent information on the use of available maltose-negative yeasts in NABLAB production and their flavour potential.

2. Rising Interest in NABLAB

Consumers' increasing interest in health issues, along with the acknowledgment of situations in which alcohol should not be consumed (driving, pregnancy, and various drug treatments), necessitate the development of a new alternative to beer. Ignoring the alcohol content, beer is a highly nutritious beverage [1]. It is a sugar-free, fat- and cholesterol-free beverage that is rich in essential vitamins and minerals (vitamin C and the group of B vitamins, and it is an important source of B-12, especially of non-animal origin, and selenium for some regional barleys) [2]. Beer also contains high levels of magnesium, antioxidant substances (e.g., polyphenols, flavonoids), and soluble fibre [2].

Another important ingredient in beer, hops, was introduced to beer production in the 9th century (822CE) [2,12,13] for its sensorial properties (bittering flavour and floral aroma) [13]. Later, the flavours (primarily bitterness and aroma) that hops imparted to beer were recognised, and its use became ubiquitous in brewing. Aside from their use in brewing beer, hops have also been used in traditional medicine since ancient times [13]. The possible beneficial properties of hops are mainly related to their polyphenols—the antioxidants they contain. Additionally, the effects of hops in reducing adipocyte differentiation, increasing nitric oxide production, and regulating oestrogen are mentioned in the literature. This latter property has led to the emergence of hop-based products as potential supplements to support menopausal women [14–17]. The levels of these compounds are quite variable due to differences in the types of hop, the region where they grow, harvest times, and other changes caused by processing and brewing [2].

Beer's calories come from its alcohol and carbohydrate content. A bottle of standard beer (350 mL) containing 4% alcohol contains approximately 105 kcal. Craft beers have 145-248 kcal/350 mL [1]. By comparison, the calories of a non-alcoholic beer with 0.4% alcohol are approximately 90 kcal/350 mL [18]. Non-alcoholic beer produced with a maltose-negative yeast contains 21 kcal per 100 mL (69.3 kcal/350 mL) [19]. On the other hand, 350 mL orange juice contains approximately 135 kcal [20].

Between 2013 and 2019, the sold production volume in the EU increased from 0.59 to 1.38 billion litres. In 2019, the share of non-alcoholic beer represented 3.8% of all beer volume. Five countries accounted for 80.8% of sold production volume: Germany, The Netherlands, Spain, Poland, and Czechia. Per capita, the average apparent consumption (2017–2019) was highest in Czechia, followed by the Netherlands, Spain, Luxembourg, and Germany [21].

To sum up, NABLAB have beneficial effects based on the healthier aspects of beer without the negative effects of alcohol. In addition to reducing alcohol consumption, NABLAB can be a good alternative to beer for consumers before or during otherwise dangerous activities (using a motor vehicle or playing sports), or under situations where alcohol would be prohibited due to health concerns (pregnancy and drug therapy). The NABLAB category of beverages also allows breweries to expand their product portfolios and lower their tax burden [10].

3. Beer and Flavour

Prior to the discovery of microorganisms and microbiology, beer (or wine) was produced by spontaneous fermentation, where non-*Saccharomyces* yeasts predominantly initiated fermentation. The resulting "beer" had less alcohol and different aromas than today's beer. In spontaneous fermentation, the microbiological source is soil, raw materials, or the production environment [22]. More recently, "beer" has evolved to include a wide variety of beverages that are brewed with water using different malts, are flavoured with diverse varieties of hops, and are fermented with generally two types of yeast: top fermenting (*Saccharomyces cerevisiae*) and bottom fermenting (*Saccharomyces pastorianus*) [23]. Beers typically range from 3% to 6% alcohol v/v, although they can go as high as 15% for certain speciality beers. Under aerobic conditions, yeast tends to proliferate, produce CO_2, and consume sugar (respiration). Under anaerobic conditions, yeasts ferment by converting pyruvate to ethanol and CO_2 [22]. In addition, yeast assimilates amino acids to synthesise new cells, enzymes, and proteins. Higher alcohols, carbonyl compounds, esters, sulphur-containing compounds, and organic acids formed as fermentation by-products define the aroma profile of beer and influence its quality [24].

The complex beer flavour combines an extensive diversity of aromas (volatile compounds), the most important of which are esters, higher alcohols, short–medium-chain fatty acids, and non-volatile substances such as iso-alpha acids (which are responsible for astringency and bitterness). Beer aroma can be made up of more than 800 different compounds, but just a few dozen of these can be aroma-active [25]. Additionally, the perception of flavour components is highly correlated with the pH and alcohol content of the beer [3]. An essential part of beer's taste and aroma substances consists of the metabolic intermediate by-products of yeast formed during fermentation. As a result, the type of yeast used and its metabolism is highly related to these taste and aroma substances [26]. In another reaction during fermentation, ethanol and higher alcohol condensates react with carboxylates, producing esters. If insufficient ester formation occurs, the flavour of the beer is negatively affected [27,28]. Numerous esters may be found in beers; of these, the most prominent are 2-phenylethyl acetate, ethyl acetate, isoamyl acetate, ethyl octanoate, and ethyl decanoate. These esters contribute fruity and flowery flavours, such as apple, citrus, blackcurrant, banana, and rose, to beer [29–31].

Higher alcohols, which are related to amino acid catabolism, are very important in beer flavour. Amino acids are significant for forming higher alcohols, such as isoamyl alcohol, isobutanol, and propanol via the Ehrlich pathway. Deaminated amino acids by transamination (α-keto acids) are decarboxylated to produce the corresponding aldehydes. These aldehydes are then further reduced to higher alcohols [10,32]. Higher alcohol aromas can be desirable at concentrations of about 90 ppm but may lead to displeasing aromas and even cause headaches at higher levels [28]. The synthesis of esters and higher alcohols is correlated with some parameters such as temperature (positively correlated), pressure (increased pressure inhibits the synthesis of esters but increases higher alcohol levels), and the pitching rate of yeast (positively correlated—a higher yeast proportion increases the concentration of esters and higher alcohols). Achieving the requisite dissolved oxygen concentration in wort is also integral to ensuring proper yeast growth and thus appropriate ester and higher alcohol levels. Higher alcohol and ester concentrations can also be affected by the type of barley used in the brewing process. An increase in sucrose or starch in wort can lead to a loss in the formation of esters and higher alcohols [33]. Additionally, pH affects esterification and higher alcohol production in the wort. Lower pH values may reduce levels of higher alcohols and increase ester levels in beer. The pH of a beer is an additional crucial parameter for ageing and quality. For microbiological safety, products with a pH lower than 4.5 (in the USA pH 4.6) can be pasteurised, while foods of a higher pH than 4.5 must be sterilised over 100 °C [34]. Beers that have pH levels above 4.0 can have some more "toasted" flavours, but in some cases, a higher pH can result in soapy or caustic notes in the beer [35,36].

During the mashing and boiling, a majority of wort aldehydes form; then, during the fermentation process, additional aldehyde formation occurs (via an anabolic process from oxo-acids and via the catabolic pathway from exogenous amino acids) [37]. Acetaldehyde is the upfront aldehyde in beer and is produced during ethanol production from carbohydrates. The amount of acetaldehyde can vary in the fermentation and maturation of beers (about 2–20 mg/L). Perpète, et al. [38] state that the dissolution of aldehydes in ethanol causes a lower perception of the wort taste, which is defined as "aldehyde retention". When the retention of aldehydes in alcoholic beers is about 32–39%, it is only 8–12% in a non-alcoholic beer [4]. Additionally, brewer's yeast can reduce the aldehyde content during re-fermentation. After primary fermentation, the beer has a low nutrient content and does not contain much dissolved oxygen. The yeast then moves towards an essentially fermentative metabolism, which reduces NADH-NADPH levels by significant amounts. Yeast will use a number of aldo-keto reductases to regenerate the oxidised forms of these coenzymes, which are known as a "yeast reducing power" [39]. Because yeast metabolism decreases aldehydes to less-aroma-active compounds in the wort due to a shorter fermentation process, the greater concentration of sugars (mono-disaccharides, such as maltose) and the absence of ethanol intensify undesirable worty flavours in NABLAB [10,38,40].These carbonyl compounds, which contribute to the potato-like flavours with very low odour thresholds, are known as methional (malt, cereal, and potato), 3-methylbutanal (malty, grainy note, and cocoa), methyl propanol (coca, ripen melon), 2-methylbutanal (cocoa), and 3-methylpropionaldehyde (wet cereal) flavours [29,41–43]. Recently, Piornos, et al. [43] identified 27 odorant-active compounds in non-alcoholic beer produced through restricted fermentation; furthermore, amongst the Strecker aldehydes involved in the formation of a "worty flavour", Piornos, et al. [43] determined that (E)-β-damascenone, 5-ethyl-3-hydroxy-4-methyl2(5H)-furanone (spicy, curry), and phenylacetaldehyde (flowery, honey-like) were the main contributors. Furthermore, 5-ethyl-3-hydroxy-4-methyl-2(5H)-furanone is associated with Maillard reactions in beer. (E)-β-damascenone can be produced by the acid-catalysed breakdown of glycosides [43,44]. According to the study of Missbach, et al. [29], this undesirable worty flavour in alcohol-free beers was more pronounced just before swallowing.

Other important off-flavours in beers and NABLAB are diacetyl (2,3-butanedione), dimethyl sulphide (DMS), and 4-vinyl guaiacol. A vicinal diketone, diacetyl, is produced due to the oxidation of acetolactate as a yeast by-product of the valine metabolism during fermentation [28]. Exhibiting a buttery taste, diacetyl has a low odour threshold, and its level may vary according to the yeast type, fermentation temperature, and production method [4,45,46]. Dimethyl sulphide (DMS) is produced by reducing dimethyl sulfoxide (DMSO) and gives unpleasant cooked vegetable and corn notes. With characteristic clove-like tones, 4-vinyl guaiacol has a distinct aroma and is produced by the decarboxylation of ferulic acid [28]. Another taste parameter that shows up a lot in NABLABs is sweetness. Standard wort contains high percentages of maltose and maltotriose as sugar but lesser amounts of sucrose, fructose, glucose, and dextrins [47]. Depending on the type of malt used, the sugar content of wort can be different [48]. Maltose, the main component of the total fermentable sugars, is between 58.6% and 72%, followed by maltotriose and glucose at 17.5–25% and 14.2–21.7%, respectively. Fructose has the lowest concentration (1.8–2.9%) [10,48–50]. The maltose/maltotriose ratio varies between 6 and 11 [48]. In NABLAB, non-fermentable maltose, maltotriose and dextrins that cannot be metabolised by yeast are left unfermented after fermentation and therefore remain in the produced beer. They add body, mouthfeel, and palate fullness to the beer, and contribute to its flavour [47]. In this context, NABLAB produced with limited sugar consumption have often been reported to have an unbalanced sweetness/bitterness ratio due to high residual sugar concentrations [3]. In a recent study of Rettberg, et al. [51], they examined the sensory properties of 19 commercial, Pilsner-type, non-alcoholic beers produced using different methods, and in general, the beers from a restricted fermentation method were considered more worty, thick, and sweet. In contrast, beers de-alcoholised by physical methods were

considered as the sourest, the thinnest, and the least sweet (with the lowest taste/aroma intensities) [51]. According to Bellut, et al. [10], all the alcohol-free beers they studied were described as "wort-like", "bread-like", or "honey-like".

3.1. Production Methods for NABLAB

Methods of producing NABLAB may be categorised into two groups: physical and biological. In the physical method, the alcohol produced by fermentation is separated from regular beer. It requires significant investments in specialised equipment to separate the alcohol (through membrane-mediated or thermal processes). After optimizing the separation process, the sensory quality of the NABLAB produced using physical methods is generally acceptable. However, aroma losses and baked-like odour differences occur when using thermal systems to aid in separation.

The advantage of physical methods is that they can separate ethanol from beer to negligible levels [4]. These methods are:

1. Thermal processes (thin-layer evaporation, continuous vacuum rectification, and falling film vacuum evaporation);
2. Membrane technologies (dialysis, reverse osmosis);
3. Extraction (solvent extraction, supercritical carbon dioxide, and adsorption extraction).

Thermal processes focus on the partial or complete ethanol removal from fully fermented beer.

In the thin-layer evaporation system, in a short time beer is passed under vacuum through a large and steam-heated centrifuged area as a thin film (0.1 mm), and ethanol is removed. Examples of thin-film evaporators, which produce a thin liquid film in a mechanical (rotational movement) way, are the Centritherm and spinning cone column systems (Flavourtech Company) [3,4]. These systems allow the rapid and efficient separation of volatile compounds, such as ethanol, from a thin liquid (beer) film [3]. The vapor flows out of the column top and passes through a condenser system, which captures the volatiles in a concentrated liquid form, and then it is given back to the de-alcoholised beer. This system is strong enough to reduce an original beer alcohol concentration from 5% to 0.01–0.03% in a single pass [4]. The thermal impact on de-alcoholised beer by the Centritherm evaporator is minimal compared to other types of evaporators because the contact time of beer to the heating surface is short (approximately 1 s) and operating temperatures are very low (35–60 °C). In other evaporation systems, contact time is often greater than 30 s. The only negative aspect is the risk of oxygen being introduced into the moving system [3].

Continuous vacuum rectification is a two-stepped technique. First, volatile compounds are separated in a vacuum degasser, then ethanol is recovered in a vacuum column about 42–48 °C, after which aroma compounds and CO2 are added back to the de-alcoholised beer [52]. But even after these volatile recovery steps, the aroma levels of this system's de-alcoholized beer were below the detection limits [3,53].

The falling film evaporation system is a similar method but does not consist of moving parts. Under vacuum conditions, beer flows down the heating tubes as a thin film because of gravity and is evaporated in a few seconds [3].

Membrane technologies consist of dialysis and reverse osmosis. All of the membrane processes have less thermal impact on beer than thermal processes, and they can automatically operate; however, they also require significant capital and running costs [4].

In dialysis, liquid components exchange through a semipermeable membrane by diffusion. In the de-alcoholisation process, the alcohol of the beer passes through the membrane into a dialysate liquid. But in addition to ethanol, a significant fraction of other low molecular weight components of the beer, such as higher alcohols, esters and short-chain fatty acids also cross the membrane [3]. When comparing with the reverse osmosis method, the non-alcoholic beer produced with this method has a normal content of CO_2 and no need for a high-pressure pump. So, the costs are lower than reverse osmosis. In the reverse osmosis process, the determining factor for the passage of substances is the pressure differential of different liquids [4].

Both thermal and membrane processes require an extra process step with additional costs. The resulting de-alcoholised beer can have a great loss of flavour, freshness, and body [3,24,28,54].

Extraction methods include solvent extraction and supercritical carbon dioxide and adsorption extraction. To extract a component from a material, there should be contact with another material or phase which has higher affinity for that particular component [28]. For the alcohol removal from a beer, it is necessary to use water insoluble solvents with high solubility in ethanol to attract beer (it has to have a high affinity, such as liquid carbon dioxide). Under some temperature and pressure conditions, the carbon dioxide acts similar to organic solvents and also removes some important aroma compounds from beer in the first phase. These compounds are added to de-alcoholised beer in the second phase. Under the different conditions of these two phases, the extraction of aroma or ethanol compounds can be managed. This method also has a high cost of operation and an additional unit for extraction to the industrial plant. The advantage of these processes is their running at room temperatures, meaning there is no thermal impact on the resulted beer [55]. In the adsorption extraction, hydrophobic adsorbents, such as zeolites with a Si:Al ratio greater than 12, are used. Beer is given through carbon dioxide in a zeolite-coated column under pressure. The beer percolates until the eluent has a low alcohol concentration. Desorption from zeolite can be done by elution with a solvent such as supercritical carbon dioxide or and organic solvent, or by heating. A separator is used to recover volatile aroma compounds that had been trapped with the ethanol. With this method, the sensorial characteristics of the non-alcoholic beer are close to its origin. However, this method requires specific additional equipment and involves high operating costs. Additionally, the adsorbent must be regenerated at the end by supercritical CO_2 and heat [28,56].

Limited ethanol formation methods (in the fermentation process) are the most common biological methods. The biological methods are as follows:

1. Arrested fermentation;
2. The cold contact process;
3. The use of unique microorganisms.

In arrested or interrupted fermentation, yeast perform a partial fermentation by using the same equipment in a beer production plant. After a point, yeast are removed from the media and fermentation stops [28]. Because it is not fully fermented, the final beer is poor in aromatic compounds and has a worty smell [28]. Generally, products produced with this method are corrected with the addition of some aroma compounds, such as isoamyl acetate (banana), to overlap the worty off-flavour [57].

As one of the most preferred biological methods, the cold contact process aims to limit alcohol formation by affecting yeast metabolism with a shorter fermentation process at low temperatures (typically just above 0 °C). However, in this method, the formation of desirable volatiles such as esters (3-methylbutyl acetate, 2-phenylethyl acetate, and ethyl acetate), higher alcohols (3-methyl-1-butanol and phenylethylalcohol), and reduction of carbonyl compounds are especially limited [43].

The difference between these "special" yeasts and conventional brewer's yeasts is based on their zero/lowered amount of alcohol production. Selecting a suitable microbial yeast strain with desirable characteristics [5] or the deliberate modification of brewer's yeast by genetic engineering and/or randomising mutation is a strategy to produce low- or no-alcohol beer [58]. With this method, there is a potential to produce a beer containing alcohol less than 0.05% v/v [59]. Genetically modified yeasts have been studied in vitro with mutant yeasts that produce more organic acids, isoamyl alcohol, and isoamyl acetate than regular yeasts [58]. Yeasts developed with the CRISPR-Cas9 technique can have exciting abilities besides producing less alcohol [22,60,61]. For example, the yeast can generate hydrogen during fermentation, which could potentially be marketable, storable, or usable as a source of energy. The enzyme pyruvate formate-lyase would redirect the carbon metabolism of yeast to formate formation, and then formate hydrogen lyase might split it to produce hydrogen and carbon dioxide [62,63]. Another example is the creation

of a yeast strain that can produce flavoured hop molecules in beer [64]. Hops are an expensive and popular additive of beer. A more controlled and sustainable alternative to hop production and harvest could be realised [22].

Generally, biological methods are produced with conventional brewing equipment and therefore do not require extra investment. Nevertheless, other NABLAB processes rely on limited ethanol production, and can require unique materials and equipment, such as a continuous bioreactor or cell immobilization carrier (continuous fermentation with immobilised yeast). On the other hand, the use of unique microorganisms (such as *Saccharomycodes ludwigii*) is seen as potentially more useful given its adaptability to traditional methods and the need for less equipment and infrastructure investment. Applying these special yeasts is not complicated from a fermentation perspective [3]. The alcohol content of beer produced by the conversion of glucose, fructose, and sucrose using specialised yeasts is often very low—about 0.5% *v*/*v* [4]. The absence of a maltose transporter and the enzyme maltase in specialised yeasts has made producing NABLABs very popular in the last few years [8–10].

3.2. Maltose-Negative Microorganisms

3.2.1. Genetics

In the past, genetic studies on maltose transport in yeasts mostly focused on *S. cerevisiae* [65–69] and later on lager yeasts [50,70–72], so our knowledge of maltose transport mechanisms in yeasts is limited to these few species. The main fermentable sugars of brewer's wort are maltose and maltotriose. Across the *S. cerevisiae* plasma membrane, the transportation of maltose is accomplished primarily by a maltose–proton symport mediated by transporters and to a lesser extent by facilitated diffusion. On the other hand, brewer's yeasts have more than one gene to carry out maltose fermentation [70]. In the literature, maltose fermentation (in most *S. cerevisiae* strains) requires at least one of the five unlinked and highly homologous *MAL* loci (*MAL1-4, MAL6*), which map to subtelomeric regions on different chromosomes. Additionally, similarly functional transporters discovered are *AGT1*(α-glucoside transporter) [73] and, more recently, *MTT1* (*MTY1*-like transporter) [50,74,75]. At least one copy from the three genes, unlike *MALx1, MALx2*, and *MALx3* genes (x indicates the locus of origin), occurs in every *MAL* locus. *Malx1p* encodes the maltose transporter, while *Malx2p* encodes an intracellular maltase (α-glucosidase), which controls splitting sugar into monomers. For *MALx1-MALx2* structural gene expression, *Malx3p* encodes a DNA-binding transcriptional activator, which is particularly essential in the existence of maltose [65–69]. Except for *MAL11* and its allele *AGT1*, all MALx1 genes show high similarity. The DNA sequence of *MAL11/AGT1* is only 57% identical to the other four *MALx1* transporter genes [50]. *MTT1* have 54% similarity with *ScAGT1* and 90% similarity with *MALx1* genes. *MPH2 (YDL247w)* and *MPH3 (YJR160c),* which were discovered by genome sequencing to be maltose transporter genes, are also mentioned, but reports are conflicting [70,71,76–79]. Brown, et al. [80] stated that these transporters could only function with turanose. Some differences were observed between ale and lager strains. Lager yeast strains, except for *MPH3* and *MAL61* transporter genes, have the *MAL11, MAL21, MAL31, MAL41, MPH2*, *AGT1*, and *MTT1* genes. This may clarify the differentiation of maltose transport kinetics in lager and ale strains [50,70]. According to the publications, maltotriose is described as a substrate for *Malx1, Agt1*, and *MTT1* genes, and is used in later periods of the wort fermentation [74]. As is to be expected, in the absence of these genes, the ability of yeasts to ferment maltose or maltotriose may be negative [81].

Brickwedde, et al. [72] studied maltose transport using CRISPR-Cas9 technology to determine how both parental genomes contribute to maltose metabolism in cold-tolerant *Saccharomyces eubayanus* and which CBS 12357T/FM1318 strains grow on maltose (not on maltotriose). In the genome sequence of *Saccharomyces eubayanus*, four open reading frames similar to the *MAL31* gene (*SeMALT1*, *SeMALT2, SeMALT3*, and *SeMALT4*) in *S. cerevisiae* were found [82]. These genes were placed at two almost identical (97% similarity) *SeMAL*

loci similar to the canonical *S. cerevisiae MAL* loci [70,83]. These four transporter genes were similar in maltose-specific transport functionality. According to the study results, the "*SeMALT1-4*" overexpression in the *S. cerevisiae* strain lacking the maltose transport gene achieved growth on maltose (but not on maltotriose). Systematic CRISPR-Cas9-assisted *SeMALT2* and *SeMALT4* deletion (MALT genes that share 99.7% similarity) eliminated the growth of *S. eubayanus* CBS 12357T on maltose, which is predominantly responsible for maltose uptake in maltose-grown cultures. Although the expression of the other two genes, *SeMALT3* and *SeMALT1*, in "Mal-negative *S. cerevisiae* strain *IMZ616*" can restore growth, low expression levels in *S. eubayanus* CBS 12357T inhibit the growth on maltose in the absence of both *SeMALT2* and *SeMALT4*. As reported in the study, *S. cerevisiae* IMZ616 is maltose-negative and originates from three *MAL* loci (*MAL1, MAL2*, and *MAL3*) containing *S. cerevisiae* CEN.PK102-3A. In the study, it was reported that these three *MAL* loci, *MPH2* and *MPH3*, alongside the *IMA1-5* and *SUC2* genes, which encode α-glucoside hydrolase, were cut out using the cas9 technique to abolish growth on α-glucosides. In the study, it was shown that the simultaneous expression of a maltase and a maltose transporter is required for the growth of a maltose-negative yeast (*S. cerevisiae* IMZ616) in maltose [72].

3.2.2. Application of Maltose-Negative Yeast Strains for NABLAB Brewing

From the consumer's point of view, yeast activity in beer fermentation is primarily linked with the quality of beer. It determines the sensory properties—such as in many other fermented products. The ability of yeasts to grow on certain substrates can be linked to specific hydrolytic extracellular enzymes, and understanding these enzymes is therefore essential for managing the fermentation process. The ability of native yeasts to secrete different extracellular enzymes may play a crucial role in the "varietal aroma" of the product [84,85]. The use of maltose-negative yeast strains with poor fermentation abilities is a biological method for NABLAB production that retains a part of the volatile elegance of conventional beers but may also be practical in the removal of a "worty" taste [86].

Brewers and researchers are working to find new commercially viable maltose-negative yeasts from alternative sources. In the case of maltose consumption, studies showed that some wine strains *of S. cerevisiae* are not well adapted to maltose fermentation because the sugars they need to use in the grape must are mostly fructose and glucose [87–89]. Some cider yeasts exhibit similar characteristics [90]. Not only wine and cider, but also other traditional fermented or fermentable goods are considered as an important source of new yeasts for the brewing industry. Potential yeast sources include: grape must (*Saccharomycodes ludwigii*) [89], kombucha (*Hanseniaspora vineae, Hanseniaspora valbyensis, Torulaspora delbrueckii, Zygosaccharomyces kombuchaensis, and Zygosaccharomyces bailii*) [10,91], traditional chicha beverages ((Andean beer) two non-conventional *Saccharomyces cerevisiae strains—ERS1 strain, EYS4 strain, and Torulaspora delbrueckii*) [84,87], and pickle (*Zygosaccharomyces rouxii*) [89]. Sourdough cultures (*Kazachstania servazzii* and *Pichia fermentans*) [6] have also investigated. Other environments that could be investigated for possible maltose-negative yeast that might be useful in brewing include: *Trigonopsis cantarellii* and *Candida sojae* [92], honey (*Zygosaccharomyces rouxii*) [89], fermented honey by-products (*Hanseniaspora uvarum, Wickerhamomyces anomalus, Zygosaccharomyces rouxii,* and *Zygosaccharomyces bailii*) [93], various exotic plants [7], and cold habitats such as Antarctica (psychrophilic yeast strains—*Mrakia gelida*) [36,94].

To summarise, the criteria for a yeast in the production of NABLAB [10] are as follows:

1. It should not be capable of fermenting maltose;
2. It should have the ability to grow in the presence of iso-α-acids, which are derived from hops;
3. It should not exhibit positive POF behaviour (phenolic off-flavours—POF1 gene absence) POFs: 4-vinylguaiacol—clove-like flavour, only acceptable in special types of beers such as Belgian/German ales [95], 4-vinylphenol—a solvent-like flavour, 4-vinylstyrene, which has a plastic-chemical like flavour [96]);
4. It should flocculate easily (strongly strain-dependent);

5. It should be consumer safe.

The sugar consumption, POF production, and flocculation potential of selected yeast species can be seen in Table 1, and a more detailed version can be found in the Supplementary Materials Table S1.

Table 1. Comparison of sugar consumption, POF production, and flocculation of selected yeast species.

Species	Maltose	Glucose	Fructose	Sucrose	POF	Flocculation (%)	References
Saccharomycodes ludwigi	-	+	+	+	-	27.1–60	*
Saccharomyces cerevisiae ERS1	-	+	+	+	+	18.5	[87]
Mrakia gelida	-	+	+	+	U	U	[36]
Trigonopsis cantarellii P-69	-	U	+	U	U	U	[92] ****
Candida sojae T-39	-	U	+	U	U	U	[92] ****
Candida shehatae	V	+	U	+	U	U	[97–99]
Candida zemplinina	-	U	U	U	U	U	[100,101]
Wickerhamomyces anomalus P-2.4	W	U	+	U	U	U	[92] ****
Kazachstania servazzi VTT C-191027	-	U	U	U	U	4	[6]
Pichia fermentans VTT C-191032	-	U	U	U	U	0	[6]
Pichia kluvyeri CBS 188	-	+	U	-	U	U	[99]
Pichia kudriavzevii	V ***	+	U	U	U	U	[102]
Cyberlindnera subsufficiens strain C6.1 CBS 5763 **	-	+	U	+	-	32 ± 1 51 ± 4	[5]
Cyberlindnera misumaiensis 837 A	-	+	U	-	-	78 ± 3	[5]
Hanseniaspora valbyensis KBI 22.1	-	+	+	+	-	11 ± 8	[10]
Hanseniaspora vineae KBI 7.1	-	+	+	-	-	41 ± 4	[10]
Torulaspora delbrueckii EGT1 KBI 22.2	-	+	+	+	-	96.2 17 ± 0	[87] [10]
Zygosaccharomyces bailii KBI 25.2	-	+	+	+	-	45 ± 0	[10]
Zygosaccharomyces kombuchaensis KBI 5.4	-	+	+	-	-	44 ± 3	[10]
Zygosaccharomyces rouxii DVBPG 4084, 6187, 6424, 6463, 6921	+/W	+	V	-	U	U	[89]
Zygosaccharomyces rouxi CBS 732	W	+	U	V	U	U	[99]
Brettanomyces bruxellensis BC02, BC07, BC11	-	+	U	-	+	U	[103]
Brettanomyces bruxellensis	V	+	U	+	+	U	[99,104]

U—undeclared; +/W—positive or weak; V—variable; POF—phenolic off-flavour; ± indicates the standard deviation. * All *S. ludwigi* data were compatible among the references. For an easier evaluation, all *S. ludwigi* data about sugar consumption, POF production, and flocculation were combined: Maltose utilisation data was obtained from [92]; VTT-C181010 [89]; DBVPG 3010, 3304, 3398, 3931, 4116, and 6771 [10]; TUM SL 17; WSL17 [36]; Glucose utilisation data was obtained from [89]; DBVPG 3010, 3304, 3398, 3931, 4116, and 6771 [10]; TUM SL 17 [36], WSL17; Fructose utilisation data was obtained from [10]; TUM SL 17; WSL17 [36]; Sucrose utilisation data was obtained from [89]; DBVPG 3010, 3304, 3398, 3931, 4116, and 6771 [10], TUM SL 17; WSL17 [36]; POF info was obtained from [10]; TUM SL 17: flocculation% info was obtained from 27.1 [6]–60 ± 7 [10]; TUM SL 17. ** According to Kurtzman, et al. [104], growth on maltose "variable" [5]. *** Consumption max 1 g/L. **** In Krogerus, et al. [92], yeast growth on various carbon sources (glucose, fructose, maltose, and maltotriose) was measured in microplate cultivations and grouped into three groups: those capable of growing on both maltose and maltotriose, those only on maltose, and those on neither. Shown as graphics: The blue areas were given here as negative. Yellow areas given here as weak usage (w). Orange and darker areas are given here positive. Note: Even though their classification is controversial, species with limited maltose usage ability have also been added to this table due to their use in low alcohol beverages (the use of maltose for these varieties is very limited).

Saccharomycodes ludwigi

The yeast *Saccharomycodes ludwigii*, which has been the subject of most studies [6,89,92,105,106], is a species used in the industry [99], and which is patented [107,108] and accepted as a reference for many NABLAB studies today [6,10,92,109]. Regardless of its close relationship to *Saccharomyces uvarum*, *S. ludwigi* does not contain maltase and invertase and cannot ferment

maltose. Thus, beer produced with this yeast strain can have a really low ethanol level and more fruity, estery flavours [36]. Using *S. ludwigii* yeast, Narziss, et al. [110] obtained a non-alcoholic beer by fermenting wort with 11.5% wt at 20 °C, and then lowered the temperature to 0 °C on the final day of fermentation. *S. ludwigii* produced 0.68% alcohol *v*/*v* after 120 h of fermentation (20 °C) [111].

In another study Liu, et al. [105] used *S. ludwigii* yeast (8.1 °P wort, 12 °C) to obtain a non-alcoholic beer containing 0.47% ethanol *v*/*v*. It was reported that higher alcohol (39 mg/L) and ester (1.9 mg/L) concentrations were low, and the resulting non-alcoholic beer was weak in aroma and had a sweet taste.

Mohammadi, et al. [106] immobilised *S. ludwigii* (DSM 3447) on brewers' spent grain (BSG) and found that *S. ludwigii* could ferment maltose partially (1.7% alcohol *v*/*v* in 6.5 °P wort at 7 °C). The authors reported that this higher amount of alcohol production than other researchers might have been due to higher enzymatic activity and lower intracellular pH values.

Mortazavian, et al. [112] also carried out fermentation studies using *S. ludwigii* (strain DSM 3447 in 6 °P wort). The fermentation trials were held at three different temperatures (4, 12, and 24 °C) and two different pitching rates (10^7 and 4×10^7 cells/mL) with different aeration periods. In the subsequent sensory evaluation of their fermented beers, the alcohol levels were between 0.15 and 1.20% *v*/*v*. The final beer products were considered inadequate due to their immature and sweet flavours when fermented at low temperatures (4 and 12 °C), and sour–lactic flavours at higher temperatures (24 °C).

De Francesco, et al. [89] examined six different *S. ludwigii* strains at 20 °C under aerobic conditions (50 mL, 12 °P wort) to determine its suitability for producing low-alcohol beer (generally isolated from grape). The alcohol concentrations of the products obtained in the experiments were between 0.51 and 1.36% *v*/*v*. In terms of aromatic compounds, esters were reported to vary between 1 and 15 mg/L, higher alcohols to be between 43 and 77 mg/L, and diacetyl (off-flavour with buttery tones) values were found to be below the threshold value of 0.1 mg/L. The yeast strain which produced the lowest alcohol level among trials was reported as a potential yeast for NABLAB production.

Bellut, et al. [10] produced a low-alcohol beer with 0.5% alcohol concentration *v*/*v* with *S. ludwigii* (6.6 °P wort, fermentation 25 °C, 3 days). The beer's ester and higher alcohol levels were low at 0.8 and 21 mg/L, respectively. In addition, the study found that diacetyl, an undesirable compound, was below the palatability threshold. It was stated in the authors' studies that non-alcoholic beers were not differentiated well according to principal component analysis (PCA), but that all of them were reported to have a wort-like smell and a sweet taste (strain: TUM SL 17). In the tasting panel, the resulting non-alcoholic beer created using *S. ludwigii* was defined as having a worty, bread-like flavour and a sweeter taste.

Although Johansson, et al. [6] reported that two trials were carried out in their studies and some differences were observed in the first and second trials, they found that beers produced using *S. ludwigii* yeast generally had a fruity/estery aroma with apple notes. These apple and pear flavours are associated with esters such as 3-methylbutyl acetate. A synergistic effect created with other esters was also detected because 3-methylbutyl acetate was reported in very low levels in all samples. It has been reported that the 2-phenylethyl acetate production of *S. ludwigii* (6.11 mg/L) is higher than the flavour threshold of 3.8 mg/L and could have a role in total aroma by making rose, honey, and apple note contributions to the final beer product.

On the contrary, in the study of Krogerus, et al. [92], *"S. ludwigii"* was described as "worty" with high levels of branched-chain aldehydes. According to this study, commercial *S. ludwigii* strain beer had the lowest scores in fruitiness, possibly because of a low monoterpene alcohol content.

Comparison of the suitability and aroma potential of some *Saccharomycodes ludwigi* yeast strains can be seen in the Table 2, and a more detailed version can be found in Supplementary Materials Table S2.

Table 2. Comparison of the suitability and aroma potential of some *Saccharomycodes ludwigi* yeast strains.

Strain	Alcohol by Volume	pH	Volatile Compounds Evaluation	Sensory Notes	References
C181010	0.43% ± 0.00	4.84	High-branched-chain aldehydes noticed.	"Worty" but also desirable fruity aromas were observed in fermented worts.	[92]
VTT C-181010	0.68%	4.78	Overproduction of 2-phenylethyl acetate than its flavour threshold.	Predominantly cereal, DMS, and sweet taste descriptors were reported, but also malty and bready tastes were found. Body described as higher than in other samples. Apple notes were reported on the nose.	[6]
TUM SL 17	0.50% ± 0.01	5.67	0.80/21.05 (E/Ha mg/L)	Described as worty, honey, bread-like, or sweet	[10]
WSL17	1.23% ± 0.02	4.60	9.35/42.23 (E/Ha mg/L)	Described as having yellow colour and a good clearance but with a weak foam head. Had apricot fruit with hop, cereal, malt, and caramel notes.	[36]
DBVPG 3010	0.51%	U	14.91/43.31 (E/Ha mg/L) DBVPG 3010 had the highest diacetyl among the other *S. ludwigis* in the study (15.77 µg/L (still below the threshold)). With its low ethanol production and appreciable higher-ester alcohol production, DBVPG 3010 was identified to be a worthwhile yeast strain for NABLAB production.	U	[89]
DBVPG3304	0.73%	U	1.56/53.85 (E/Ha mg/L) Did not find properly due to low pleasant volatile compounds production.	U	[89]
DBVPG 3398	0.72%	U	1.21/47.66 (E/Ha mg/L) Did not find properly due to low pleasant volatile compounds production.	U	[89]
DBVPG 3931	1.24%	U	2.35/62.34 (E/Ha mg/L) This strain produces a high amount of higher alcohols but also the alcohol amount is higher than other *S. ludwigis*.	U	[89]
DBVPG 4116	1.36%	U	4.15/76.62 (E/Ha mg/L) This strain produces a high amount of higher alcohols but also alcohol production is higher than other *S. ludwigis*.	U	[89]
DBVPG 3054	0.70%	U	2.06/42.99 (E/Ha mg/L) Did not find properly due to low pleasant volatile compounds production.	U	[89]
U	0.68%		1.82/45.30 (E/Ha mg/L) Diacetyl 140 (µg/L)	U	[110]
CSIR-NCL 3261	1.2% ± 0.02	5.04	U	U	[109]
U	0.47%		1.9/39 (E/Ha mg/L) concentrations were found low	U	[97,105]

Table 2. *Cont.*

Strain	Alcohol by Volume	pH	Volatile Compounds Evaluation	Sensory Notes	References
DSM 3447	1.7% ± 0.06	3.47	U	U	[106]
DSM 3447	0.15–1.20%	U	U	The treatments with *S. ludwigii* in 24 °C produced lactic acid and showed lactic sour attributes. In 4 °C and 12 °C it had an undesirable sweet and immature flavour.	[112]

As observed from all these studies, *S. ludwigii* is generally a valuable yeast for obtaining a low-alcohol beer product. This yeast strain is currently used in industry and is considered as "safe".

The suitability and aroma potential of specific maltose-negative yeasts for NABLAB brewing can be seen in Table 3. A more detailed version of this table can be seen in the Supplementary Materials Table S2.

Table 3. Comparison of the suitability and aroma potential of selected maltose-negative yeast species.

Species	Alcohol by Volume	pH	Volatile Compounds Evaluation	Sensory Notes	References
Saccharomyces cerevisiae ERS1 EYS4	1.28–1.29%	4.58–4.60	ERS1's acetaldehyde amount was reported to be close to its flavour threshold. Ester production was reported higher in all Ecuadorian strains. (ERS1, EYS4) compared to reference strains.	U	[87]
Mrakia gelida Re-fermented DBVPG 5952	1.40% ± 0.12	4.71	3.516/27.7 (E/Ha mg/L)	Reported as being fruity with apricot, grape, and litchi descriptors, but also with hop, cereal, malt, and caramel notes. A better fruity olfactive intensity, compared to the beer made with *S. ludwigii*. In visual assessment, the beer was defined as having poor head foam, yellow colour, and good clarity.	[36]
Trigonopsis cantarellii P-69	0.14% ± 0.00	4.81	Reported to contain high amounts of the desired monoterpene alcohol and trans-geraniol, and low amounts of aldehydes.	The lowest aldehyde levels (off-flavour) were reported in beer fermented with *T. cantarellii*. Additionally, its performance was found comparable to a commercial *S. ludwigii* reference strain.	[92]
Candida sojae T-39	0.22% ± 0.01	4.72	Reported to contain notably higher amounts of the trans-cis geraniol, which has a desirable rose note.	Notes of diacetyl were reported on the sensory panel in *C. sojae* beer. However, its performance was found comparable to a commercial *S. ludwigii* reference strain.	[92]
Candida shehatae CICC 1766	0.37–0.47%	U	Reported that the beer produced contains a high amount of ester and a low amount of diacetyl (below 0.05 mg/L).	Reported that the produced non-alcoholic beer does not have the typical worty and sweet taste seen in non-alcoholic beers produced with limited fermentation. Had a flavour similar to regular alcoholic beer.	[97,98]

Table 3. *Cont.*

Species	Alcohol by Volume	pH	Volatile Compounds Evaluation	Sensory Notes	References
Candida zemplinina Y.01667 Y.01670	1.5%	4.8	U	In mixed fermentation trials with *S. cerevisiae* (in all fermentation media tested), *C. zemplinina* was reported as the most successful starter for non-alcoholic beer production with desired sensory properties.	[100,101]
Wickerhamomyces anomalus P-2.4	0.60% ± 0.00	4.64	*W. anomalus* beer was reported as having the highest volatile ester concentrations.	Reported that *W. anomalus* beer was not liked because solvent-like tones were detected in smell and taste. It has been reported that this is due to ethyl acetate, typically produced at higher levels by *W. anomalus.*	[92]
Kazachstania servazzi VTT C-191027	0.73%	4.78	Production of 2-phenyl ethyl acetate at concentrations above the flavour threshold has been reported.	Reported that the descriptors of cereal, DMS, and sweet were dominant in sensory analysis, but pear and apple notes were also detected. It has been noted that *K. servazzii* beer is characterised by a sweet aroma typical for low-alcohol beers.	[6]
Pichia fermentans VTT C-191032	0.52%	4.79	A higher amount of 2-phenylethanol production (2.77 mg/L) and a lower amount of acetaldehyde (2.34 mg/L) than other studied yeast strains (*K. servazzii* and *S. Ludwigi*).	Stated that the beer had a cinnamon- and clove-like spicy/phenolic flavour reminiscent of Belgian and German beers fermented by "POF+" yeasts. The taste has been described as original and pleasant. In addition to these, it has been reported that *P. fermentans* beer has lower DMS and cereal values than other samples, and contains notes of banana and melon.	[6]
Pichia kluvyeri PK-KR1	0.1%	U	25/20 (E/Ha)	Reported that its flavour is very close to a beer with alcohol content at least 4% (*v*/*v*). Sensory examination has reported higher amounts of desired esters, such as phenylethyl acetate and isoamyl acetate, and lower amounts of undesired acids, such as decanoic and octanoic acids, in beer.	[97,110]
Pichia kudriavzevii Pk129	0.5–0.8%	U	50/50 (E/Ha mg/L) *P. kudriavzevii* found as producing relatively more desired volatiles in a balance.	U	[102]
Cyberlindnera subsufficiens C6.1	0.36% ± 0.00	4.45	12.8/9.8 (E/Ha mg/L)	Described as pleasantly fruity (banana, pear, maracuja, and mango) and has also been reported to have a bit of a worty-like character in sensory examination.	[5]
Hanseniaspora valbyensis KBI 22.1	0.35% ± 0.01	4.84	0.9/23.3 (E/Ha mg/L) For isoamyl alcohol values it was exhibiting the highest (16.5 mg/L) in study. Unwanted diacetyl levels were above the flavor threshold in light beers.	Reported to have a "cereal-like" character and the diacetyl flavour was felt by half of the panellists.	[10]

Table 3. *Cont.*

Species	Alcohol by Volume	pH	Volatile Compounds Evaluation	Sensory Notes	References
Hanseniaspora vineae KBI 7.1	0.34% ± 0.02	4.78	6/20.2 (E/Ha mg/L)	Defined as sweet, must-like, and honey-like, with black tea and caramel tones.	[10]
Torulaspora delbrueckii EGT1	1.32% ± 0.01	4.65	High ester production. Had the best E:HA ratio in studied yeast strains (*S. cerevisiae* ERS1 and EYS4).	U	[87]
Torulaspora delbrueckii VTT C-191036	0.8–1.0%	4.60–4.75	*T. delbruecki* strains have been reported to be effective reductants, reducing branched-chain aldehydes by more than 90% compared to wort.	U	[6]
Torulaspora delbrueckii KBI 22.2	0.5% ± 0.01	4.69	0.77/18.1 (E/Ha mg/L) It was reported that non-alcoholic beer produced with *T. delbrueckii* KBI 22.2 contained the least amount of isoamyl alcohol (10.4 mg/L) among other samples in the study.	Described as cereal-like honey-like, wort-like, and bread-like by all panellists. Half of the panellists detected the unpleasant diacetyl flavour.	[10]
Zygosaccharomyces bailii KBI 25.2	0.42% ± 0.07	4.71	1/23.1 (E/Ha mg/L)	Reported that it has the best potential among the yeasts studied in brewing non-alcoholic beer with its improved sensorial character.	[10]
Zygosaccharomyces kombuchaensi s KBI 5.4	0.48% ± 0.01	4.61	1/22 (E/Ha mg/L) Unwanted diacetyl levels were above the flavour threshold in light beers.	Described as honey-like, wort-like, and bread-like. Unwanted diacetyl notes detected by the half of the panellists.	[10]
Brettanomyces bruxellensis LTQB6	4%	U	According to study, produced a higher acetaldehyde concentration than threshold value.	U	[113]
Zygosaccharomyces rouxii DVBPG 4084, 6921, 6187	0.93–1.63%	U	Reported that *Z. rouxii* strains produced the highest amount of volatile compounds, especially higher alcohols as well as diacetyl and acetaldehyde, because it produces ethanol at high levels (more than 33.78/92.07 (E/Ha mg/L). DVBPG 6187 has the highest diacetyl with 851.40 (µg/L).	U	[89]

± indicates the standard deviation; U—undeclared. E/Ha indicates esters/higher alcohols ratio (mg/L).

Saccharomyces cerevisiae

Famous *Saccharomyces* species are used to produce traditional beer types and many fermented products in the food industry. To produce a better sensorial-characterised NABLAB, there are many studies on *Saccharomyces* strains with different phenotypic and genotypic profiles. The *Saccharomyces cerevisiae var. chevalieri* strain is commercially used in NABLAB production throughout the brewing industry [114].

Grijalva-Vallejos, et al. [87] evaluated the suitability of some selected yeasts for beer production that were isolated from chicha beverages. Two maltose-negative Ecuadorian *S. cerevisiae* strains (ERS1 and EYS4) in the study were found to be "POF+", and the *S. cerevisiae* ERS1 strain showed some flocculation. It should be noted that Ecuadorian strains are generally not good at flocculation (generally under 20%). All chichas in the study were noted to contain 3-methylbutanol (≥82 mg/L) at levels higher than the sensory threshold value (≥70 mg/L). It was stated that all the strains used in the study had good

ester production, and among these strains, ERS1 was characterised as having the best "higher alcohol: ester" balance. The beers created using ERS1 and EYS4 were found to have exceeded the recommended higher alcohols (HA): esters ratio (4–4.7:1), with an acetaldehyde concentration close to the threshold (25 mg/L) [87].

Mrakia gelida (psychrophilic yeast strain)

These basidiomycetous yeasts strains are not very common worldwide and are generally considered as low/non-fermenting yeasts due to their higher sensitivity to ethanol [36]. Tsuji, et al. [115] studied the ethanol toleration and production amounts of some *Mrakia* strains. *Mrakia gelida* (*M. gelida*) is a psychrophilic yeast that has a very low ability to use maltose (lower than *S. ludwigii*) [36,94]. According to the literature, low-temperature fermentation can have a positive impact on end-product flavour: as in the production of wine, the higher preservation of primary (comes from original material—varietal aroma) and secondary (fermentation) aromas can be obtained at low temperatures of 10–15 °C rather than 25 °C, possibly due to less evaporation occurring for varietal aromas, as well as an increased secondary metabolism occurring for acetate and ethyl esters at lower temperatures [36,116,117]. In the Thomas-Hall, et al. [94], "*Mrakia* spp" yeasts (*M. blollopis, M. frigida, M. robertii, and M. gelida*) isolated from Antarctica were utilised to produce beers using a standard kit for home brewers with a commercial malt extract (5% sucrose, 6–15 °C). However, it was reported that the fermentation stopped when the alcohol concentration rose above 2% (*v*/*v*), and the fermentation of sucrose could not be completed.

In another study, De Francesco, et al. [36] examined the promising strain *Mrakia gelida* DBVPG 5952 in a comparative study with a commercial starter of *S. ludwigii* WSL17. Fermentations were completed successfully, but the resulting ester and higher alcohol amounts were considered low. The authors found that the "yeast reducing power" of *M. gelida* may be high because the content of off-flavour-associated aldehydes in the beer was lowered in re-fermented samples. Aldehyde methional (an off-flavour responsible for worty or cooked vegetable flavours) levels in experimentally examined beers were lower than the threshold [42]. It was also reported that the diacetyl content in the sample beers was well below the detection threshold. Diacetyl is commonly found in unconventional yeast beers, and some cases may not be reduced to the derivatives of diacetyl, which are flavourless (such as 2,3-butanediol and acetoin) [111,118]. According to the sensory analysis results of the De Francesco, et al. [36] studies, the *M. gelida*-fermented sample was described as clear, yellow in colour, fine-headed, and persistently foamy. The aroma profile of the beer produced with *M. gelida* gave better results, especially in terms of fruity olfactive intensity, compared to the beer made with *S. ludwigii*. It was reported that the sensory parameters of the raw materials used in the study were similar between the beers produced with the two yeast varieties. Sweetness was reported as low in both samples. Additionally, bitterness was determined to be very low in both samples, with a resulting positive effect on the drinkability of both beers. As a result, *M. gelida* was considered a good brewer's yeast candidate for NABLAB production. In terms of security, Thomas-Hall, et al. [94] reported that in their laboratory safety studies with the beers produced with *Mrakia* strains, there were no abnormalities in the laboratory rats. *Mrakia* strains cannot grow at the human body temperature due to their psychrophilic nature and therefore cannot be considered as pathogenic for humans and animals.

Trigonopsis cantarellii (syn. *Candida cantarellii*)

C. cantarellii strains are from the microbiota of wine-related environments and may have their origins in vineyards, winery-environment equipment, and bottling lines [119]. Krogerus, et al. [92] similarly used *T. cantarellii* isolated from a brewery location to evaluate its potential for low-alcohol beer production. In this study, *T. cantarellii* (strain P-69) was reported to produce lower amounts of off-flavours (the lowest aldehyde levels) and significantly higher amounts of trans-geraniol, a desirable monoterpene alcohol compared to the non-alcoholic commercial reference beer sample. This yeast cannot grow at the human body temperatures of 37 °C. With these characteristics, the strain is considered to have low pathogenicity. The sensory analysis concluded that beers made with *S. ludwigii*

and *T. cantarellii* were similar to commercial full-strength beer [92]. In a different study, it was reported that *T. cantarellii* could also produce trace amounts of 4-ethylphenol in synthetic environments [120].

Although "*T. cantarellii*" was listed as a safe yeast by Bourdichon, et al. [121], it is not listed as a QPS/GRAS biological agent [122] for industrial use, so it must also be tested further to ensure its safety.

Candida sojae

Candida sojae is a xylose-consuming yeast from the Saccharomycetales order of Ascomycota [123]. Recently Krogerus, et al. [92] studied the use of *C. sojae* (strain T-39), typically considered a contaminant, in the production of low-alcohol beer. It was reported that both diacetyl (butter, off-flavour) and dimethyl sulphide (cooked corn, off-flavour) were detected at concentrations close to flavour thresholds in beers produced with *C. sojae* yeast. Diacetyl (butter, off-flavour), which is known to be both indirectly produced by yeast during fermentation and degraded over time, is typically an indicator of an overly short fermentation time. Panellists also noted diacetyl in the sensory analysis of the beer produced with *C. sojae*. Overall, it was reported that beer produced with *C. sojae* performed comparably to that produced with commercial *S. ludwigi*. Both beers were found to be fruity, mild, and sweet.

For process safety, both the tolerance of *C. sojae* to well-known food antifungals and the potential for biofilm formation were evaluated in the study. According to the results, the pathogenicity of *C. sojae* was low because the strain could not grow at body temperatures around 37 °C. In addition, this strain was reported to produce biogenic amines only in trace levels less than in commercial beer. However, *C. sojae* is not listed in GRAS/QPS biological agents safety lists [122], so the strain should be examined more thoroughly for industrial use.

Candida shehatae

Candida shehatae is also a xylose-fermenting yeast and produces ethanol [99,124,125]. In their patent, Li, et al. [98] proposed using *Candida shehatae* for non-alcoholic beer production. The *C. shehatae* strain CICC 1766 (9 °P, 14 °C, 300 mL trial) produced 0.47% ethanol *v*/*v*, and the amount of undesired diacetyl was found to be less than 0.05 mg/L. According to the study's results, the non-alcoholic beer made using *C. shehatae* had a high content of esters and did not have the worty and sweet taste of typical non-alcoholic beers. In higher volume production (200 L), non-alcoholic beer was produced quite successfully with an ethanol content of 0.37% *v*/*v*. The resulting beer's flavour was similar to regular beer and contained a diacetyl level of less than 0.05 mg/L [97,98].

Candida zemplinina (Starmerella bacillaris)

Candida zemplinina (syn., *Starmerella bacillaris* [126]) is a fructophilic, psychrotolerant, acidogenic, and highly osmotolerant non-*Saccharomyces* microorganism that can easily be found in grapes and musts [127]. *C. zemplinina* cannot ferment maltose, galactose, and lactose, but can ferment glucose, sucrose, fructose, and raffinose [128]. In the study of Estela-Escalante, et al. [101], the *C. zemplinina* strain was evaluated for craft beer production with different wort conditions (350 mL laboratory scale, 12 P wort, pH 4.8, at 18 °C, 8 days). The alcohol value of the beer produced only with *C. zemplinina* was reported to be 1.5% *v*/*v*, but no sensory results were reported [100,101]. In mixed fermentation trials with *S. cerevisiae*, and all fermentation media tested, *C. zemplinina* was the most successful starter yeast for the production of an organoleptically pleasant, low-alcohol beer (mainly the CZ3 strain) [129]. *C. zemplinina* was reported as a safe microorganism that may function as a biocontrol agent for various food pathogens [130].

Wickerhamomyces anomalus (formerly *Pichia anomala*)

While some studies indicate that *Wickerhamomyces anomalus* (*Pichia anomala*) is unable to metabolise maltose [131], others report that some strains of *W. anomalus* can utilise maltose [132]. The common belief is that *W. anomalus* can produce high levels of some higher alcohols and esters, such as phenyl ethanol, 2-phenylethyl acetate, ethyl propanoate, and particularly ethyl acetate [92,133]. These compounds are metabolically important

due to their antifungal properties and, although varying in concentration, can take on fruity-solvent-like characters that affect the flavour and aroma profile of the beer.

Successful results can be obtained with an appropriate strain selection. For example, Osburn, et al. [134] reported that *W. anomalus* (strain YH82) could produce successful sour beer with fruit notes such as apple, pear, and apricot.

Canonico, et al. [135] studied the *W. anomalus* strain DiSVA 2 alone and mixed with other strains (maltose-positive). According to the results, a single *W. anomalus* trial produced 1.53% alcohol *v*/*v* (12.3 °P, 19 °C, 500 mL scale) and also 0.17 g/L lactic acid (pH 4.75). In all mixed fermentations, ethyl acetate and ethyl butyrate were reported to be high, and acetaldehyde was reported as low. The study concluded that all mix fermentation trials were positively affected by the addition of *W. anomalus*. However, a sensory analysis was not performed in this study.

According to the recent study results of Krogerus, et al. [92], beer produced using *W. anomalus* yeast showed high ester levels. However, undesirable solvent-like notes were found in the beer produced by *W. anomalus*, and as a result, it was not liked in terms of smell and taste. These solvent-like notes were suggested to be caused by ethyl acetate, a typically abundant compound of the *W. anomalus*.

Wickerhamomyces anomalus was listed as a safe yeast (for beneficial use) in the study of Bourdichon, et al. [121]. However, it is not listed as being a QPS or GRAS biological agent [122].

Kazachstania servazzii

Not only part of the *Saccharomycetaceae* family, the genus *Kazachstania* is also the most closely related to *S.cerevisiae* on an evolutionary scale and is naturally found in grapes/musts [136]. There have been some recent oenologic interests in *Kazachstania servazzii* due to its aromatic nature. For example, in a study by Lin, et al. [137], it was reported that *K. servazzii* produced a fruity wine with a high levels of esters—specifically banana/isoamyl acetate. In another study, the sequential inoculation of Shiraz and Merlot must with *Kazachstania spp*. followed by *S. cerevisiae* was studied [136]. According to results of this study, *Kazachstania* spp. significantly increased the level of acetate esters—predominantly isoamyl acetate and phenylethyl acetate—in both Shiraz and Merlot wines.

Correlated with these results, in the study of Johansson, et al. [6], the level of 2-phenylethyl acetate (rose, honey, apple sweetish flavour) in beer produced with *K. servazzii* yeast exceeded the threshold value (3.8 mg/L) and was detected as 7.39 mg/L. The flavour profiles of beers made with *S. ludwigii* and *K. servazzii* were reported to be similar, and sensorial descriptors were reported to be "DMS" (dimethyl sulphide worty off-flavour at high concentrations), "Cereal", and "Sweet". These sensorial descriptors are generally the main components of low-alcohol beers produced with maltose-negative yeasts. According to the study, in addition to the fruity/estery aroma of all samples in the study, "*K. servazzii*" also has unique notes of pear and apple [6]. In the study, the "body" of the beer produced with *K. servazzii* yeast was defined as high, while its "sweetness" was defined as low. The authors of the study argued that *K. servazzii* yeast performs as well as *S. ludwigii*. In addition, the cold tolerance of *K. servazzii* yeast is considered to be a potential advantage when compared to *S. ludwigii* for industrial-level brewing. Along with psychrotolerance, the *K. servazzii* used for brewing lager beers at lower temperatures has increased microbial stability. *K. servazzii* also reduces wort aldehydes more effectively than *S. ludwigii*.

For safety purposes, although the *Kazachstania telluris* strain was reported as pathogenic [138], there is no pathogenic evidence for *K. servazzii*. More importantly, *K. servazzii* cannot grow at body temperatures such as 37 °C, which reduces the risk of pathogenicity for humans.

Pichia fermentans

In the study by Johansson, et al. [6], the aroma profile of beer produced with *Pichia fermentans* yeast is unexpectedly different from beers made with *S. ludwigii* and other yeasts. According to this study, the beer produced with *P. fermentans* yeast has a pleasant "phenolic and clove-like" taste and is positively described as "original". In a sensory

analysis of beer made with *P. fermentans*, a unique spicy–phenolic flavour was identified. This "cinnamon- clove-like" flavour is typical of German and Belgian beers fermented with "POF+" species. This taste is considered a positive feature that may be used in producing weizen and farmhouse-style non-alcoholic beers [139]. Beers produced with *P. fermentans* have lower "DMS" and "grain" values compared to *S. ludwigii* beers as well. Additionally, although all samples in the Johansson, et al. [6] study had a "fruity/estery" aroma, the *P. fermentans* sample was distinguished from others by its banana and melon flavours. In another article, *Pichia fermentans* was reported to increase the terpene concentration in wine through "glycosidase activity". Similar increases in beer flavour occurred through the release of glycosidically-bound hop terpenes [140]. This glycosidase activity may be responsible for fruity flavours such as melon. These flavours cannot be explained by the volatile profile of beer produced with *P. fermentans* yeast. The study also reported that the "sweetness" of beer produced with *P. fermentans* was lower than the sweetness of beer produced with *K. servazzii*—likely due to the masking effect of phenol notes.

Regarding safety, *P. fermentans* yeast is not considered to be potentially pathogenic, since no clinical cases associated with *P. fermentans* fermentation and human pathogenicity have been seen, and the yeast cannot tolerate temperatures as high as 37 °C. Food-sourced *Pichia fermentans* is listed by Bourdichon, et al. [121] as a safe yeast for beneficial use in their study.

Pichia kluyveri

Pichia kluyveri has the ability to ferment quickly, produces a moderate amount of esters (particularly phenylethyl acetate and isoamyl acetate), and generates large amounts of higher alcohols. This yeast also produces lower levels of volatile unwanted acids (especially decanoic and octanoic acids). As a result, this yeast is considered a promising variety for producing new low-alcohol beers [97,133,141]. *P. kluyveri* also has the ability to produce volatile thiols in Sauvignon Blanc. Most notably, *P. kluyveri* creates 3-mercaptohexan-1-ol and 3-mercaptohexyl acetate resembling a grapefruit and passion fruit aroma, respectively [142]. In a patent application, *Pichia kluyveri* (PK-KR1, PK-KR2 strains) were used to produce NABLAB [143]. In a 1000 L-scaled beer production, the wort gravity was 8.3 °P (62% barley, 38% wheat), and the fermentation was held at 20° for three weeks. The resulting beer had a very close flavour profile to a regular beer, with at least 4% ethanol v/v. With its limited ability to ferment wort sugars (only glucose), *Pichia kluyveri* can effectively convert hop compounds into positive aroma compounds when producing NABLAB beers [10].

Pichia kluyveri was also listed by Bourdichon, et al. [121] as safe in their study. However, it is not listed as a QPS/GRAS biological agent [122].

Pichia kudriavzevii

Lately, *P. kudriavzevii* has attracted interest for many fermented products [102,144–146]. In the studies of van Rijswijck, et al. [102], a (12 °P wort) with 24 strains of *Pichia kudriavzevii* produced beers at 0.5–0.8% alcohol v/v because of limited maltose utilization. The relative ratio of esters to higher alcohols was reported to be 50:50, a little higher than *C. fabianii* (40:60).

Dos Santos et al. [145] also studied the application of two *P. kudriavzevii* strains (*P. kudriavzevii* BB1-BB2) for craft beer production. Because the alcohol levels of the beers from the study were above 4% v/v, these strains likely can ferment maltose. Sensory descriptors of P. *kudriavzevii* BB1-BB2 beers were alcoholic, white wine–cider, ripe fruit–fruity, and slightly acidic. The study concluded that these strains could play a useful role in inoculation (but not individually) for the bioflavouring of craft beers.

P. kudriavzevii was listed as safe yeast (for the beneficial use) in the study of Bourdichon, et al. [121], but it is not listed as a QPS or GRAS biological agent [122].

Cyberlindnera Yeasts

The use of yeasts such as non-*Saccharomyces Cyberlindnera* are known for their high ester production (e.g., *Cyberlindnera saturnus*—formerly *Williopsis saturnus—strain NCYC 500*). This yeast's ability to mask the worty flavour of non-alcoholic beer produced through

limited fermentation has been noted as promising [5,147]. Low-alcohol beer produced with *C. mrakii*, and having an ethanol content of 1.7% *v*/*v* (400 mL, 13.8 °P, 21 °C for 14 days), was compared with beer produced with "*S. cerevisiae* brewer's yeast (Safale US-05)" containing 6.9% *v*/*v* ethanol. Ethyl and isoamyl acetate concentrations of beer fermented with *C. mrakii*, which has limited fermentation ability, were 20 times higher than beer fermented with brewer's yeast. According to the results of the study, the authors produced isoamyl acetate, which has a banana-like aroma, in very high concentrations using "*Cyberlindnera* spp." Although Liu, et al. [147] recommended using strain NCYC 500, they expressed concern that higher ethyl acetate production would result in a solvent-like off-taste in beer.

van Rijswijck, et al. [102], studied non-traditional yeast performances (49 wild yeasts, including nine *Cyberlindnera fabianii* isolates) in co-culture with brewer's yeasts (100 mL, single inoculation to a barley wort extract—12 °P, 20 °C, 7 days). Ethanol in *C. fabianii* beer was 0.6% *v*/*v*, while the ratio of esters to higher alcohols was relatively high, reported as approximately 40:60 (esters: alcohols). For *S. cerevisiae* isolates, this ratio has been reported as 15:85 (esters: alcohols).

In the study by Bellut, et al. [5], three species were found to be "maltose +"—able to utilise maltose (*Cyberlindnera fabianii*, *C. jadinii*, and the *Cyberlindnera mrakii*—formerly *Williopsis saturnus var. mrakii*) —and three as "maltose -" (*Cyberlindnera misumaiensis*, *C. subsufficiens*, *and Cyberlindnera subsufficiens).* According to the study, non-alcoholic beer containing *Cyberlindnera misumaiensis*, a maltose-negative strain, was described as solvent-like and unpleasant. In contrast, beer from a *Cyberlindnera subsufficiens* strain was found to be more fruity compared to the reference commercial non-alcoholic beers and was defined as promising [5]. Some *Cyberlindnera* strains, such as *C. jadinii* and *C. mrakii*, are in the safe list of the study of Bourdichon, et al. [121], but more study is needed for other strains.

Hanseniaspora valbyensis

The genus *Hanseniaspora* is one of the main yeast groups isolated from grapes. *Hanseniaspora valbyensis* is generally isolated in a kombucha environment [10,148,149].

Bellut, et al. [10] stated in their study that beer produced with *Hanseniaspora valbyensis* yeast contains the highest amount of isoamyl alcohol (16.5 mg/L—fruit-like, brandy-like) amongst beers of other varieties (threshold value 50–70 mg/L) [32]. Although the threshold value cannot be exceeded, aroma substances can affect the total fruitiness by acting synergistically. On the other hand, it was reported that the undesirable diacetyl flavour (0.21 mg/L) was higher than the threshold of 0.1 mg/L. Beer produced with *H. valbyensis* yeast had a "cereal-like" character, and the diacetyl aroma was felt by half of the panellists [10].

Hanseniaspora vineae

Hanseniaspora vineae (anamorph *Kloeckera africana*) is a non-*Saccharomyces* yeast used in winemaking. In the co-fermentation with *S. cerevisiae*, it has the potential to improve the aromatic properties of wines by increasing the level of ethyl acetate and 2-phenylethyl acetate [150–152]. In Bellut, et al. [10], the sensory analysis results (principal component analysis—PCA) showed no significant difference between the beer produced with *Hanseniaspora vineae* yeast and other non-alcoholic beers. *H. vineae* beer (also other non-alcoholic beers), in general, was defined as sweet, must-like, and honey-like, with black tea and caramel tones.

Torulaspora delbrueckii

Torulaspora delbrueckii, which was formerly called *T. vafer*, *T. fermentati*, and *T. rosei*, as well [153], is a commonly isolated yeast in fermented products. Various strains of *T. delbrueckii* are recognised as highly cryo- and osmotolerant [154]. Therefore, *T. delbrueckii*, which has a phylogenetic affinity with *S. cerevisiae*, is seen as a very promising yeast strain, especially in biotech industries that involve wine or bread fermentation [155,156]. Additionally, in wines, *T. delbrueckii* was one of the first *non-Saccharomyces* strains commercially applied as Zymaflore® Alpha, PreludeTM, BiodivaTM, Primaflora® VB BIO, and Vinifer NSTD [155]. It has been shown in studies that *Torulaspora delbrueckii* can produce

high levels of amyl alcohol and 2-phenylethanol (fruity flavours) [133,157]. In their study, Tataridis, et al. [158] compared *T. delbrueckii* with a *S. cerevisiae* strain (top fermented) to produce a German wheat beer. This beer was reported to be a high ester beer with rose, bubble gum, and banana flavours, which the panellists preferred.

In a study of beers produced using 28 *T. delbrueckii* strains, the resulting beers were described as fruity, citric, and full-bodied in sensory analysis (alcohol 2.66% *v*/*v*) [156].

Michel, et al. [111] studied 10 *T. delbrueckii* strains from different habitats. According to their results, the yeast strains could not ferment maltose/maltotriose, yet they still created good flavours so that they may be suitable for low-alcoholic beer production.

The authors also mentioned another strain that is capable of fermenting wort sugars to 4% alcohol *v*/*v* with a pleasing floral and fruity taste (because of large amounts of amyl alcohol (64.83 mg/L) and 2-phenylethanol (23.7 mg/L)). Academic studies seem to offer highly variable results for *T. delbrueckii* [99,156,158], but it is nevertheless well-known in the wine industry to impart a more fruity character in wines [99].

In the study of Bellut, et al. [10], the lowest isoamyl alcohol concentration (10.4 mg/L) was detected in the case of *T. delbrueckii*. However, there were otherwise few differences between *T. delbrueckii* and other strains. In general, beers produced with *T. delbrueckii* were described as having worty smells and a sweet taste [10].

In a different study, Grijalva-Vallejos, et al. [84] isolated this yeast from chicha, a traditional beer from Ecuador. The focus on this yeast as a potential cultivar in beer production is that among non-*Saccharomyces* yeast species, it can produce fewer off-flavours and more significant amounts of desirable compounds in beer/wine fermentations [87]. The acetaldehyde concentration was close to the sensory threshold value (25 mg/L) in beer produced by *T. Delbrueckii* (D291 strain) [87]. All of chicha's 3-methyl-butanol concentrations ($\geq$82 mg/L) were found to be above the threshold of 70 mg/L. Regarding ester production in the study, 2-phenylethyl acetate could only be detected in chicha by *T. delbrueckii* EGT1, and chichas by EGT1 and D291 strains were reported to have the highest ester levels. According to the study, the *T. delbrueckii* strain EGT1 showed even better growth than *S. cerevisiae* strains in colder conditions. The sensitivity for ethanol in the *T. delbrueckii* EGT1 strain may relate to its low viability, which also limits fermentation. The higher alcohol (HA): ester ratio (4–4.7: 1) [159] recommended in the study was best obtained in beers produced with *T. delbrueckii* EGT1, and it was seen that the differences between the strains were mainly related to higher alcohol production. In the study, *T. delbrueckii strains* could be grouped because of the production of ethyl acetate in chichas and ethyl decanoate in beers.

T. delbrueckii is listed by Bourdichon, et al. [121] as a safe yeast for beneficial use.

Zygosaccharomyces bailii

Because of *Z. bailii*'s high tolerance to preservatives and other stressors, it was regarded as a spoilage yeast [129]. In the study of Bellut, et al. [10], non-alcoholic beer produced with *Z. bailii* had a moderate fruity and worty odour and a sweet taste as compared to other alcohol-free beers. In sensory analysis (principal component analysis—PCA), all alcohol-free beers were described as having a sweeter taste and a worty odour. Additional characteristics such as "light grassy", "fruity", and "white wine" were also descriptors for *Z. bailii* beers in the sensory analysis [10]. Among the yeast isolated from kombucha, *Z. bailii* KBI 25.2 strain was selected as a potential indigenous yeast strain that could be used for low-alcohol beer production.

In another study, Capece, et al. [129] studied the environment's effects on starter behaviours using different commercial substrates to produce various beer styles (Pilsen, amber, and weizen). A variety of wild yeasts (*Zygosaccharomyces bailii, Torulaspora delbrueckii, Candida zemplinina*) and a selected *S. cerevisiae* strain were tested. Mixing the *Z. bailii* strain with *C. zemplinina* strains (P4-CR1) was found to be less effective for the reduction of ethanol, as it generated the lowest reduction of alcohol content in the weizen (WW) and Pilsen (PW) fermentations. Mixed starter beers also had a higher amount of alcohol than beer made with a single starter (wild *S. cerevisiae* strain—P4) in the amber (AW) beer fermentation.

Zygosaccharomyces kombuchaensis

Zygosaccharomyces kombuchaensis, an ascosporogenous yeast, was isolated from kombucha tea [160]. *Z. kombuchaensis* was reported, along with its diacetyl production, in the study by Olaniran, et al. [161]. In beer produced using *Z. kombuchaensis*, buttery flavoured diacetyl levels were reported to be 0.15 mg/L, higher than the threshold of 0.1 mg/L [161]. According to the PCA of the sensorial panel outcomes, although no significant differentiation could be detected between non-alcoholic beers, it was observed that all of them, including the beer produced with *Z. kombuchaensis*, were defined as sweet in taste and worty in odour. Additionally, it was reported that the high diacetyl values detected in the volatile analysis of the beer produced with *Z. kombuchaensis* were detected by 50% of the sensory panellists [10].

Zygosaccharomyces rouxii (partial inability to ferment maltose)

Z. rouxii has a limited ability to ferment maltose, is a variety that can consume ethanol under aerobic conditions [162], and produces active aroma compounds [89]. Sohrabvandi, et al. [163] evaluated the osmotolerant and halotolerant food yeast *Z. rouxii* in a sequential application after *Saccharomyces cerevisiae* with the aim of producing a non-alcoholic beer. The authors observed an excellent reduction in alcohol, but the taste was compromised. De Francesco, et al. [89] similarly investigated *Z. rouxii and S. ludwigii* to produce low-alcohol beers. Unlike in the Sohrabvandi, et al. [163] study, *Z. rouxii* strains were reported to be inappropriate for low-alcoholic beer production due to their high ethanol production, but *S. ludwigii* was stated to have potential for NABLAB production. In the De Francesco, et al. [89] study, three strains of *Z. rouxii* (DBVPG 6463, DBVPG 6424, and DBVPG 6921) consumed more sugar than the other strains. It was suspected to be the result of the partial ability of the yeast to metabolise maltose [89].

From a safety point of view, *Z. rouxii* is listed by Bourdichon, et al. [121] as a safe yeast for beneficial use.

Kluyveromyces marxianus

K. marxianus (CBS6014) was reported as being maltose-negative in the study of Struyf, et al. [164]. Although this study examined the low-FODMAP production of whole-wheat bread with *K. marxianus* yeast, the results provide insight into the flavouring potential of this yeast. The study states that when *K. marxianus* CBS6014 was used in a single inoculation, 2-phenylethyl acetate, ethyl octanoate, benzaldehyde, gamma nonalactone, and 4Z-decen-1-ol were produced significantly at different levels when compared with a *S. cerevisiae* bakery strain. Unfortunately, the differences were not detected in sensory analysis. *K. marxianus* was also reported as having a high potential for industrial aroma production from phenylalanine to yield 2-phenylethanol by bioconversion [165]. Rollero, et al. [166] reported that these yeasts might contribute to complexity in wines but may also impair fermentation if competition for nutrients occurs with *S. cerevisiae*. In the same study, *K. marxianus* was also reported to have fermentations characterised by phenylethyl acetate and phenylethanol production. In another study, Rollero, et al. [167], sequentially inoculated *K. marxianus* (strain IWBT Y885) and two other non-conventional wine yeasts in order to examine the nitrogen metabolism for the purpose of modulating wine aroma. According to the results, this yeast variety can produce phenyl ethanol and phenyl ethyl acetate, close to *S. cerevisiae*, depending on the amount of phenyl alanine in the environment. In addition, highly volatile sulphur compound (VSC) production resulting from H_2S metabolism through sequential fermentation occurred when *K. marxianus* with *S. cerevisiae* were combined, and nutrients were added.

(Alcoholic fermentation of Sauvignon blanc (4.5 L glass bottles) and Shiraz (10 L buckets) wines was carried out at 15 ○C and 25 ○C, respectively, according to white and red wine production techniques).

Brettanomyces bruxellensis

It is reported in the literature that "*Brettanomyces bruxellensis*" (teleomorph *Dekkera bruxellensis* [168]) is a yeast crucial for spontaneous fermentation and is used in the mixed fermentation of Belgian-type gueuze and lambic beers [168,169], some sour English beers, and Berliner weißbier [99,170]. The typical flavour of *B. bruxellensis* can be described as horsey-or-horse, blanket, mousy, medicinal, or leather-like due to phenols (especially 4-ethyl guaiacol and 4-ethyl phenol). It is also considered to be fruity due to high ester

production. Interestingly, *B. bruxellensis* has the ability to ferment some complex carbohydrates such as maltodextrins (i.e., maltopentaose and maltotetraose), which causes a high attenuation and may have a role in the dry mouthfeel of beers where *B. bruxellensis* is used [168]. The use of sugar for this yeast varies considerably [99,103,104,171]; however, the alcohol content of the beer obtained, even in maltose-negative yeasts, seems to be too high for NABLAB production [113,172]. In addition, another point to be considered is the careful selection of the strain to be used, as some strains of *B. bruxellensis* have the potential to produce biogenic amines, biologically dangerous substances [173].

Table 4, shows the imported volatile compounds of beers produced with some maltose-negative yeasts for craft beer production, and Table 5 includes those for special beer-type production. Their more detailed versions can be found in the Supplementary Materials Table S3 and S4, respectively.

3.3. Safety

Microorganisms are ancient food processing agents that can transform and preserve food. On the one hand, a characteristic taste and texture are achieved with the development of different metabolites. On the other hand, food with a longer shelf-life is formed [176]. In this context, traditional food cultures are accepted as traditional ingredients of foods, and their use in the EU is legally permitted. However, not all yeasts are harmless. For example, *Candida tropicalis* is a pathogen (opportunistic) and has the ability to ferment maltose [177]. Maltose-negative yeasts can be isolated from many different sources other than foods. From a safety standpoint, fermented food systems are considered more acceptable than non-food strains as sources of microorganisms. Many food-system yeasts already hold "Qualified Presumption of Safety" (QPS) or "Generally Recognized as Safe" (GRAS) status [6]. The list of yeast species GRAS/QPS for food is very short. Along with *Saccharomyces* species (*S. cerevisiae, S. pastorianus, and S. Bayanus*), the QPS list of EFSA contains 17 species: *Candida cylindracea, Debaryomyces hansenii, Hanseniaspora uvarum, Kluyveromyces lactis, Kluyveromyces marxianus, Komagataella pastoris, Komagataella phaffi, Ogatae angusta, Lindnera jadinii, Schizosaccharomyces pombe, Wickerhamodoculia anomalus, Xanthophyllomyces dendrorhous, Yarrowia lipolytica*, and *Z. rouxii* [122,178]. A similar safety list is the GRAS list of safe microorganisms listed by the United States Food and Drug Administration (FDA) [178,179]. Additionally, the European Food and Feed Cultures Association (EFFCA) and the International Dairy Federation (IDF) have been carrying out since 2002 a project on the safety presentation of microbial food cultures [180].

In general, there is a severe lack of information about the use of unconventional yeasts in industry. However, yeasts not mentioned on safe lists (QPS/GRAS) are not necessarily unsafe, and there are many explanations for the absence of these yeasts from safety lists.

A strain may not have been investigated by the relevant authorities or it simply may not have been investigated regardless of use, environment, and condition. Safety assessment tests generally include analyses such as genome-based assessments, biogenic amine production, toxin production, and antibiotic susceptibility tests [176]. Even though the evaluation of food culture strain requests were given to the EFSA, only the EU Commission may conclude which cases need a pre-market safety and approval assessment.

Table 4. Comparison of selected volatile compounds in beers produced with some maltose-negative yeasts for NABLAB craft beer production (mg/L).

Compound	***S. ludwigii* VTT-C181010**	***S. ludwigii* WSL17**	***T. cantarellii* P-69**	***C. sojae* T-39**	***Cyberlindnera subsufficiens* C6.1**	***H. valbyensis* KBI 22.1**	***H. vineae* KBI 7.1**	***Z. bailii* KBI 25.2**	***Mrakia gelida* DBVPG 5952**
Acetaldehyde	4.9	0.92 ±0. 1	3.7	2.25	10.55	3.3 ± 0.4	4.1 ± 0.4 a	4.9 ± 1.3	2.30 ± 0.18
n-Propanol	2.35	2.6 ± 0.0	3.4	1.95	2.20	2.1 ± 0.1 a	2.2 ± 0.0	0.56 ± 0.03	5.7 ± 0.4
Isobutanol	6.1	13.03 ± 1.1	2.3	7.2	3.60	4.8 ± 0.1	4.6 ± 0.3	5.7 ± 0.1	9.8 ± 0.2
3-Methyl-1-butanol	U	14.3 ± 0.8	U	U	U	U	U	U	6.0 ± 0.3
3-Methyl-2-butanol	U	14.40	U	U	U	U	U	U	U
2-Methyl-1-butanol	U	5.5 ± 0.2	U	U	U	U	U	U	1.4 ± 0.1
Furfuryl alcohol	U	U	U	U	U	U	U	U	U
2-Phenylethanol (Phenyl alcohol)	8.1	6.8 ± 0.7	0.75	1.85	U	U	U	U	2.6 ± 0.1
Isoamyl alcohol (3-methylbutanol)	14.65	U	1.4	13.25	4.00	16.5 ± 1.1	13.4 ± 0.1	14.8 ± 0.2	U
Ethyl acetate	1.5	9.3 ± 0.3	<0.59	<0.59	12.00	0.90 ± 0.05	6.00 ± 0.14	1.00 ± 0.00	0.6 ± 0.1
Isobutyl acetate	<0.06	U	<0.06	<0.06	U	U	U	U	U
Isoamyl acetate	<0.14	0.03 ± 0.00	<0.14	<0.14	0.80	<0.1	<0.1	<0.1	ND
Phenyl ethyl acetate	<0.4	U	<0.4	<0.4	U	U	U	U	U
Ethyl hexanoate	<0.02	0.011 ± 0.002	<0.02	<0.02	U	U	U	U	0.009 ± 0.001
Ethyl octanoate	<0.4	0.009 ± 0.002	<0.4	<0.4	U	U	U	U	0.006 ± 0.001
Ethyl formate	U	U	U	U	U	0.78 ± 0.06	0.76 ± 0.03	0.56 ± 0.03	U
Furfural	U	0.01 ±0.0	U	U	U	U	U	U	0.007 ± 0.00
Methional	U	0.006 ± 0.3	U	U	U	U	U	U	0.009 ± 0.00
2-Methylbutanal	U	0.002 ± 0.0	U	U	U	U	U	U	0.001 ± 0.00
3-Methylbutanal	U	0.006 ± 0.0	U	U	U	U	U	U	0.007 ± 0.00
Hexanal	U	0.0007 ± 0.0	U	U	U	U	U	U	0.001 ± 0.00
Phenylacetaldehyde	U	0.01 ± 0.0	U	U	U	U	U	U	0.009 ± 0.00
Diacetyl (2,3-Butanedione)	U	0.008 ± 0.0	U	U	<0.01	0.21 ± 0.03	0.05 ± 0.01	0.03 ± 0.00	0.008 ± 0.00

Table 4. *Cont.*

Compound	***S. ludwigii* VTT-C181010**	***S. ludwigii* WSL17**	***T. cantarellii* P-69**	***C. sojae* T-39**	***Cyberlindnera subsufficiens* C6.1**	***H. valbyensis* KBI 22.1**	***H. vineae* KBI 7.1**	***Z. bailii* KBI 25.2**	***Mrakia gelida* DBVPG 5952**
2,3-Pentanedione	U	U	U	U	<0.01	U	U	U	U
Notes and references	30 L scale [92]	25 L [36]	30 L scale [92]	30 L scale [92]	60 L Scale [5]	2 L Scale [10]	2 L Scale [10]	2 L Scale [10]	25 L [36]

U—undeclared; ND—not Detected; ± indicates the standard deviation.

Table 5. Comparison of selected volatile compounds in NABLAB beers produced with some maltose-negative yeasts for the production of special beer types (mg/L).

Compound	***S. cerevisiae* ERS1**	***T. delbruckii* T10/T13**	***K. servazzi* C-191027**	***P. fermentans* VTT C-191032**	***H. uvarum VTT* C-191029**	***W. anomalus* DiSVA2 *****
Acetaldehyde	16.04± 1.60	U	3.76 ± 0.08	2.34 ± 0.00	1.8± 0.1	49.3 ± 2.8
n-Propanol	6.68 ± 0.16	3.03/4.13	2.14 ± 0.06	0.27 ± 0.01	0.7 ± 0.0	22.5 ± 0.9
Isobutanol	U	2.36/3.56	U	U	U	10.8 ± 1.3
Isoamyl alcohol (3-methylbutanol)	33.63 ± 1.11	25.26/29.10 **	17.01 ± 0.3	8.65± 0.02	16.3 ± 0.4	51.6 ± 4.1
2-Phenylethanol (β-Phenyl ethanol)	5.78 ± 0.31	15.83/13.9	ND	2.77 ± 0.09	5.1 ± 0.1	0.00 ± 0.00
Ethyl butyrate	U	U	U	U	U	0.040 ± 0.042
Ethyl acetate	4.76 ± 0,26	2.23/5.33	0.83 ± 0.03	ND	1.0 ± 0.1	1.7 ± 0.7
Isoamyl acetate	U	U	U	ND	U	0.051 ± 0.049
Phenylethyl acetate	0.20 ± 0.01	U	7.39 ± 0.19	ND	ND	0.061 ± 0.035
3-Methylbutylacetate	0.67 ± 0.06	U	0.02 ± 0.002	0.27± 0.01	0.01 ±0.00	U
Ethyl hexanoate (ethyl caproate)	0.18 ± 0.03	U	ND	ND	ND	0.056 ± 0.014
Ethyl octanoate (Ethyl caprylate)	0.27 ± 0.09	U	ND	ND	ND	U
Ethyl decanoate	0.06 ± 0.01	U	ND	ND	ND	U
Diacetyl	U	0.13/0.1	U	U	U	U

Table 5. *Cont.*

Compound	***S. cerevisiae* ERS1**	***T. delbruckii* T10/T13**	***K. servazzi* C-191027**	***P. fermentans* VTT C-191032**	***H. uvarum VTT* C-191029**	***W. anomalus* DiSVA2 *****
Ethyl formate	U	U	U	U	U	U
4-Vinylguaiacol	U	U	U	U	U	U
2-Methylbutanol	11.01± 0.36	U	4.07 ± 0.08	2.10 ± 0.02	4.5 ± 0.1	U
2-Methylpropanol	15.28± 0.30	U	7.46 ± 0.2	4.87 ± 0.08	15.0 ± 0.4	U
Linalool	U	U	U	U	U	0.110 ± 0.010 a
α-Terpineol	U	U	U	U	U	0.022 ± 0.001
Hexanoic acid	U	0.16/0.20	U	U	U	U
Octanoic acid	U	0.36/0.57	U	U	U	U
Decanoic acid	U	0.67/1.13	U	U	U	U
Notes and references	Volatiles-in-beer data was obtained from the Supplementary Materials of the article [87]. It can have a potential use in certain special beer production due to its POF(+) character.	* Bavarian wheat beer or special beer production [111](2 L).	Lager-type yeast (10 L scale) due to clean aroma and cold tolerance (authors' suggestion) [6].	Wheat beer-type (10 L scale) due to clove-like aroma of 4-vinyl guaiacol (Belgian–German ale type) [6].	The distinctive clove-like aroma of 4-vinylguaiacol was reported (Belgian–German ale type) (2 L scale) [6].	* 500 mL scale [135]. **** Sour beer production.

U—undeclared; ND—not detected; ± indicates the standard deviation. * Brettanomyces/Dekkera yeasts (mostly maltose-positive) are used for the production of Bavarian wheat beers (Hefeweizen); however, some maltose-negative yeasts have also been considered for brewing, such as *Torulaspora delbrueckii* and *Wickerhamomyces anomalus* [174]. For example, due to its complex aroma profile, *T. delbrueckii* was used in traditional wheat beer (Hefeweizen) production and was found to have potential [158,175]. ** Given as total amyl alcohols; *** W. anomalus DiSVA2 could utilise maltose weakly, total alcohol amount of its beer is 1.53% *v*/*v*; **** Osburn, et al. [134] suggest *W. anomalus* for sour beer production (strain YH82).

4. Conclusions

This study displays information about maltose-negative yeasts that have attracted attention recently, and reveals their aroma potential. These yeasts cannot ferment maltose due to deficiencies in their maltose transporters and maltase enzymes, so they are very promising for NABLAB production. Through the use of these yeasts, the organoleptic problems of NABLABs can be eliminated and new flavours can be obtained without additional investment in special equipment. Maltose-negative yeasts are the subject of even more research as successful and different results are obtained in the production of NABLAB. More successful NABLAB production in different types and characteristics will be possible with studies that will focus on new yeast varieties in the coming days.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation8060273/s1, Table S1: comparison of sugar consumption, POF production, and flocculation of selected yeast species; Table S2: comparison of the suitability and aroma potential of selected maltose-negative yeast species; Table S3: comparison of selected volatile compounds in beers produced with some maltose-negative yeasts for NABLAB craft beer production (mg/L); Table S4: comparison of selected volatile compounds in NABLAB beers produced with some maltose-negative yeasts for the production of special beer types (mg/L).

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