Beyond *Saccharomyces pastorianus* for modern lager brews: Exploring non-*cerevisiae* *Saccharomyces* hybrids with heterotic maltotriose consumption and novel aroma profile

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Non-domesticated, wild *Saccharomyces* yeasts have promising characteristics for beer diversification, particularly when used in the generation of *de novo* interspecific hybrids. A major motivation for the current work was the question whether attractive novel *Saccharomyces* interspecific hybrids can be created for the production of exotic lager beers without using the genomic resources of the ale yeast *Saccharomyces cerevisiae*. Importantly, maltotriose utilization is an essential characteristic typically associated with domesticated ale/lager brewing strains. A high-throughput screening on nearly 200 strains representing all eight species of the *Saccharomyces* genus was conducted. Three *Saccharomyces mikatae* strains were able to aerobically grow on maltotriose as the sole carbon source, a trait until recently unidentified for this species. Our screening also confirmed the recently reported maltotriose utilization of the *S. jurei* strain D5095T. Remarkably, *de novo* hybrids between a maltotriose-utilizing *S. mikatae* or *S. jurei* strain and the maltotriose-negative *Saccharomyces eubayanus* strain CBS 12357 displayed heterosis and outperformed both parents with regard to aerobically utilizing maltotriose as the sole carbon source. A high-throughput screening on nearly 200 strains representing all eight species of the *Saccharomyces* genus was conducted. Three *Saccharomyces mikatae* strains were able to aerobically grow on maltotriose as the sole carbon source, a trait until recently unidentified for this species. Our screening also confirmed the recently reported maltotriose utilization of the *S. jurei* strain D5095T. Remarkably, *de novo* hybrids between a maltotriose-utilizing *S. mikatae* or *S. jurei* strain and the maltotriose-negative *Saccharomyces eubayanus* strain CBS 12357 displayed heterosis and outperformed both parents with regard to aerobically utilizing maltotriose as the sole carbon source. 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Favorable fruity esters were produced, demonstrating that the novel hybrids have the potential to add to the diversity of lager brewing.
Introduction

Brewing has been connected to human activity for millennia. The long-term unintentional domestication of baker's yeast (mainly ale yeasts of the species *Saccharomyces cerevisiae*) has developed its superior fermentation capabilities and the pleasant mixture of aroma and flavor compounds in ale-style beers. Moreover, standardizing and regulating the brewing process in the medieval times to be conducted at lower temperatures (ca. 12°C) gave rise to lager-style beers with their distinguished clean, crisp, and refreshing character (Hornsey, 2003). The yeast responsible for lager fermentation was identified to be different from *S. cerevisiae* and named *Saccharomyces pastorianus*, a hybrid between an ale *S. cerevisiae* and the cold-tolerant *Saccharomyces eubayanus*. The latter parental wild yeast has only been discovered a decade ago (Libkind et al., 2011). *S. eubayanus* strains have been isolated from different regions of the world (Libkind et al., 2011; Bing et al., 2014; Peris et al., 2014; Gayevskiy and Goddard, 2016; Nespolo et al., 2020). Two of those isolates have also been used by the brewing industry to brew novel types of lager beer (Lovin Dublin, 2018).

Lager beer brewed with the hybrid yeast *S. pastorianus* makes up the majority of global beer volume; however, preferences toward novel specialty beers with exotic aroma profiles led to the exponential increase of craft breweries (Garavaglia and Swinnen, 2017; Jaeger et al., 2020). Industrial *S. pastorianus* strains are genetically related and therefore exhibit limited phenotypic diversity including a narrow range of flavor/aromatic profiles (Dunn and Sherlock, 2008; Okuno et al., 2015; Gallone et al., 2019; Salazar et al., 2019). Therefore, there has been a high interest in increasing aromatic complexity in lager beer by exploiting the biodiversity of wild yeasts of the *Saccharomyces* clade either individually or after hybridization with other species at the relative cold fermentation temperatures. Indeed, interspecific hybridization techniques have been a powerful tool in this context, particularly since hybrids could outperform both parental species due to heterosis effects as previously demonstrated for de novo *S. cerevisiae* x *S. eubayanus* crosses (Hebly et al., 2015; Krogerus et al., 2015; Mertens et al., 2015). Such a novel hybrid with enhanced brewing and aromatic properties has recently been commercialized for the production of lager beer (Lallemand Brewing, 2022).

The *Saccharomyces* clade currently comprises eight recognized species from various sources and locations: *S. cerevisiae*, *S. paradoxus* (Batshinskaya, 1914), *S. mikatae* (Naumov et al., 2000), *S. eubayanus* (Libkind et al., 2011), *S. kudriavzevii* (Naumov et al., 2000), *S. uvarum* (Beijerinck, 1898), *S. arboricola* (Wang and Bai, 2008), and the most recently identified *S. jurei* (Naseeb et al., 2017). Non-domesticated (wild) representatives of the genus have already shown their fermentative and desired fruity aroma potential in lager brewing with several examples recently reported for *S. eubayanus* (Eizaguirre et al., 2018; Lovin Dublin, 2018; Mardones et al., 2020), *S. paradoxus* (Nikulin et al., 2020b), and *S. jurei* (Giannakou et al., 2021; Hutzler et al., 2021). Such studies have been commonly restricted to a single or few tested strains.

Alongside the potential benefits for aroma complexity, there are a few typical traits in wild yeasts that are regarded undesired in lager brewing. These include the production of phenolic off-flavors such as 4-vinyl guaiacol (4-VG; Gallone et al., 2016), lack of cold tolerance (Nikulin et al., 2018), suboptimal flocculation (Hutzler et al., 2021), and most importantly, inefficient fermentation of the most abundant sugars in brewer's wort, maltose and maltotriose (Steensels and Verstrepen, 2014; Methner et al., 2019; Nikulin et al., 2020a). Maltotriose is typically utilized at the end of the fermentation (Dietvorst et al., 2005). Residual amounts of the trisaccharide could affect both flavor and ethanol yield, an important economical parameter to produce lagers (Zheng et al., 1994). Maltotriose utilization in yeast is complex, being determined by MAL loci harboring three gene families: MAL encoding an oligosaccharide permease, MALS encoding an α-glucosidase and MALR encoding a regulator responsible for the transcriptional induction of MALT and MALS by maltotriose (Charron et al., 1989; Brouwers et al., 2019b).

In general, non-conventional (wild) yeast species of the *Saccharomyces* clade are thought not to be able to grow on maltotriose as the sole carbon source (Mtt⁺ phenotype). This also holds for the species *S. eubayanus* since all strains known so far are unanimously maltotriose-negative (Mtt⁻) (Mardones et al., 2020). In fact, efficient utilization of maltotriose (Mtt⁻ phenotype) and particularly its fermentation to ethanol was assumed to be generally restricted to the *S. cerevisiae* and *S. pastorianus* maltotriose-positive (Mtt⁺) strains at the time when the current study was initiated. The ability of the natural hybrid yeast *S. pastorianus* to utilize maltotriose by fermentation was initially attributed solely to the *S. cerevisiae* parent (Hebly et al., 2015; Brickwedde et al., 2018). Later, it was demonstrated that the genome of the Mtt⁺ parent, *S. eubayanus*, contributed as well to this phenotype possibly due to neofunctionalization of the MAL genes (Baker and Hittinger, 2019; Brouwers et al., 2019b) or regulatory cross-talk between the two parental genomes (Brouwers et al., 2019a).

Interestingly, a few strains of the *S. eubayanus* species...
including the well-studied CBS 12357⁷ have demonstrated evolvability to maltotriose utilization (Baker and Hittinger, 2019; Brouwers et al., 2019b). These results suggest that *S. eubayanus* might be able to provide the genetic resources for a Mtt’ phenotype in novel hybrids even without *S. cerevisiae* as a parent. This approach would be particularly promising when using a second wild-type strain that also brings along certain genetic resources enabling maltotriose utilization.

In this study, high-throughput screening (HTS) for growth on maltotriose as the sole carbon source was applied to a wide range of a *Saccharomyces* isolates and led to the identification of several previously undescribed non-domesticated wild isolates with an Mtt’ phenotype. Newly identified wild strains were hybridized with the cold-tolerant *S. eubayanus* CBS 12357 leading to novel non- *cerevisiae* *Saccharomyces* hybrids. We evaluated the growth performance of the de novo hybrids on maltotriose in comparison to the established domesticated *S. pastorianus* CBS 1513 strain. We also tested the potential of the new hybrids to ferment brewer’s wort and brew beer with novel aroma profiles under conditions simulating lager brewing in lab scale.

### Materials and methods

#### Strains, plasmids, and media composition

*Saccharomyces* parental strains for the HTS for growth on maltotriose and *S. pastorianus* strains were selected from various geographical locations and origins. The complete list of strains screened for this study is shown in Supplementary Table S1 and the strains selected for further use in hybrids are summarized in Table 1. Yeasts were routinely grown on YPD medium (1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) glucose) and kept in 25% (w/v) glycerol stocks at ~80°C. Selection on Hygromycin B⁷ was performed via the addition of 300 μg mL⁻¹ (final concentration).

Experiments assessing growth on maltotriose were conducted in either YM medium containing 2% (w/v) maltotriose (YM + Mtt) or synthetic medium (SM) according to Verduyn et al. (1992) with 2% (w/v) maltotriose (SM + Mtt) adjusted to pH 6.5 with 2 M KOH; 2% (w/v) agar was added in solid media. Pre- and intermediate cultures were performed in SM containing 2% (w/v) glucose and cells were washed with SM without carbon source. Wort fermentations were conducted in industrial wort diluted to 12 °P supplemented with 0.6 mg L⁻¹ ZnSO₄·7H₂O final concentration; sugar content is listed in Supplementary Table S8. To sporulate yeast, potassium acetate (KAc) medium was used containing 2% (w/v) potassium acetate, 0.22% (w/v) yeast extract, and 0.05% (w/v) glucose adjusted at pH 7 with 1 M HCl or 2.5 M NaOH after which 0.087% (w/v) synthetic complete medium powder (Supplementary Table S1) and 2.5% (w/v) agar were added.

#### General molecular biology techniques

Primers used in this study are listed and detailed in Supplementary Table S2. PCRs for amplifying deletion cassettes, verification, and species-specific identification were performed using OneTaq® DNA Polymerase (New England BioLabs, Germany) or BIOTAQ™ DNA Polymerase (Bioline, UK), Phusion® High-Fidelity DNA Polymerase (New England BioLabs, Germany) was used to amplify fragments for Gibson assembly according to the respective manufacturer’s protocol. Colony PCR was applied *via* initially suspending cells in 0.02 M NaOH and incubating at 95°C for 10 min and using the supernatant as a DNA template.

Gibson assembly reactions composition and transformation protocols were adapted from the Gibson Assembly® Master Mix instructions (New England BioLabs, Germany) in which 15 μl of Gibson assembly master mix (1.33×; Supplementary Table S1) was used instead of 10 μl and LB medium instead of SOC medium. Transformation of deletion cassettes in *Saccharomyces* was conducted as described in Gietz et al. (1995) except for the use of lower heat shock temperatures – 37°C for 5 and 20 min applied on *S. mikatae* and *S. jurei* strains, respectively.

#### Screening of *Saccharomyces* strains for growth on maltotriose

*Saccharomyces* strains were initially tested for growth on maltotriose *via* the phenotyping on solid medium (PHENOS) pipeline from Barton et al. (2018). PHENOS produces reproducible proxy growth curves based on automatic absorbance measurements on the colony thickness subtracting the agar absorbance of a rectangular array plate fitting up to 96 strains with four technical replicates. To unequivocally confirm the species designation of the array glycerol stocks, ITS sequencing was performed (White et al., 1990). Strains from Arrays I and II (Supplementary Table S1) were analyzed for growth on YM + Mtt at 30°C. Strains from array III (Supplementary Table S1) were analyzed for growth on SM + Mtt at 25°C. Maltotriose was initially supplied from Sigma-Aldrich, UK with >90% purity and added to the YM solid media. Subsequently to decrease impurities, maltotriose was obtained from Glentham Life Sciences, UK, with ≥95% purity and was used instead for further experiments.

Mtt’ candidate strains were tested for growth in liquid SM + Mtt. Initially, 3 ml SM 2% (w/v) glucose pre-cultures were inoculated with single-cell colonies in triplicates in 10 ml glass tubes at 25°C and 250 rpm for ~16 h. Intermediate 3 ml SM 2% (w/v) glucose cultures were prepared by inoculating at 0.2 OD₆₀₀ and incubating at 25°C at 250 rpm for 24 h. Cells were washed once with SM without carbon source and once with SM + Mtt before inoculating the final 0.7 ml SM + Mtt culture at 0.2 OD₆₀₀ with two technical replicates in Krystal 24-well clear bottom microplate (Porvair Sciences, Leatherhead, UK) cultivated in...
TABLE 1 List of relevant Saccharomyces strains ([*S. pastorianus* (*Sc* × *Se*), *S. cerevisiae* (*Sc*), *S. eubayanus* (*Se*), *S. mikatae* (*Sm*), *S. jurei* (*Sj*)) used in this study.

| Strain name | Species | Location | Origin | Genotype | Reference | Notes |
|-------------|---------|----------|--------|----------|-----------|-------|
| UWOPS83-883-2 | *S. cerevisiae* | Navassa Island | *Drosophila* sp. | Wild type | UWOPS database |
| C6.1 | *S. cerevisiae* | Cameroon | Pam wine | Wild type | Stringini et al. (2009) |
| DBVPG 6881 | *S. cerevisiae* | United States | Ridge Winery | Wild type | DBVPG database |
| CBS 1462 | *S. cerevisiae* × *S. cerevisiae* | United Kingdom | Brewery | Non-engineered | CBS database |
| CBS 1513 | *S. cerevisiae* × *S. cerevisiae* | Denmark | Carlsberg brewery | Non-engineered | CBS database |
| CBS 1483 | *S. cerevisiae* × *S. cerevisiae* | Netherlands | Heineken brewery | Non-engineered | CBS database |
| CBS 12356 | *S. cerevisiae* | Argentina | Southern beech | Wild type | Libkind et al. (2011) |
| NBRC 10997 | *S. cerevisiae* | Japan | Decayed leaf | Wild type | NBRC database |
| NBRC 11002 | *S. cerevisiae* | Japan | Decayed leaf | Wild type | NBRC database |
| LSYS65-1 | *S. cerevisiae* | China | Bark tree | Wild type | CGMCC database |
| D5005 | *S. mikatae* | France | Bark tree | Wild type | Naseeb et al. (2017) |
| NG1 | *S. jurei* | France | France | *ho::HPH* | This study |
| NG16 | *S. jurei* | France | France | *ho::HPH MAT a* | This study |
| NG17 | *S. jurei* | France | France | *ho::HPH MAT a* | This study |
| Q183 | *S. mikatae* | France | France | *ho::HPH MAT a* | Hinks (2018) |
| Q184 | *S. mikatae* | France | France | *ho::HPH MAT a* | Hinks (2018) |
| NG87 | *S. mikatae* | France | France | *ho::HPH* | Derived from NBRC |
| NG88 | *S. mikatae* | France | France | *ho::HPH* | Derived from NBRC |
| NG89 | *S. mikatae* | France | France | *ho::HPH* | Derived from NBRC |
| NG90 | *S. mikatae* | France | France | *ho::HPH MAT a* | Derived from NBRC |
| NG91 | *S. mikatae* | France | France | *ho::HPH MAT a* | Derived from NBRC |
| NG92 | *S. mikatae* × *S. jurei* | France | France | *ho::HPH* | Derived from NBRC |
| NG101 | *S. mikatae* × *S. jurei* | France | France | *ho::HPH* | Derived from NBRC |

| Strain name | Species | Location | Origin | Genotype | Reference | Notes |
|-------------|---------|----------|--------|----------|-----------|-------|
| NBRC 11002 | *S. mikatae* | France | France | *ho::HPH* | Derived from NBRC |

UWOPS (University of Western Ontario Plant Sciences); CBS (Centraal bureau voor Schimmelcultures); NBRC (Biological Resource Center, NITE); CGMCC (China General Microbiological Culture Collection Center); DBVPG (Dipartimento di Biologia Vegetale, Università di Perugia). Perugia).

the Growth Profiler 1,152 (Enzyscreen, Netherlands) at 200 rpm and 25°C. The Growth Profiler determines the density of the cultures by measuring pixel density from images taken every 40 min of individual wells correlated to equivalent OD₆₀₀ values via a calibration curve with the equation of the best fit line: OD₆₀₀ = 6.1761.10 × Green value²⁸. The calibration curve was based on *S. cerevisiae* CEN.PK113-1A and was applied to generate OD₆₀₀ values for all strains tested for a comparative analysis.

**Hybrid construction and confirmation**

Interspecific hybrids were constructed by firstly disrupting the *HO* gene in the parental strains to abolish mating-type switching and self-mating and obtain stable haploids ([Strathern et al., 1982]). The cassettes to integrate the *HPH* hygromycin marker in *S. jurei* D5005, *S. mikatae* NBRC 10997, *S. mikatae* NBRC 11002, and *S. mikatae* LSYS65-1 were amplified from pAG32 (Goldstein and Mccusker, 1999), pUC18 _ho::HPH_NBRC_ 10,997, pUC18 _ho::HPH_NBRC_ 11,002, and pUC18 _ho::HPH_ LSYS65-1, respectively, (Supplementary Table S1). Homology arms with 45 bp and 500 bp were applied on *S. jurei* and *S. mikatae* strains, respectively, targeting the beginning of the *HO* open reading frame (ORF) to disrupt it and generate strains NG1, NG87, NG88, and NG89.

Sporulation and tetrad dissection were conducted via standard protocols ([Sherman and Hicks, 1991]) to obtain stable haploids confirmed to be either MAT "a" or "α" via mating-type PCR (Supplementary Table S2) and halo assay ([Julius et al., 1983]). Stable haploids were not successfully isolated from NG88 (*S. mikatae* NBRC 11002 _ho::HPH_) and NG89 (*S. mikatae* LSYS65-1 _ho::HPH_). Interspecific hybrids NG92 (*S. cerevisiae* × *S. jurei*) and NG101 (*S. cerevisiae* × *S. mikatae*) were constructed via mass mating as described by Sherman and Hicks (1991). Successful hybrid formation was confirmed via initially re-streaking the cell mass for single colonies three times after which they were sporulated to confirm tetrad formation. The production of spores demonstrated their
hybrid nature favoring sporulating hybrids in the verification process. Two and four biological replicates of $Se \times Sj$ and $Se \times Sm$ hybrids, respectively, were successfully isolated. Sporulation efficiency was determined for twenty tetrads per hybrid resulting in ~1% spore viability for each hybrid tested and glycerol stocks were randomly selected and designated as NG92 ($Se \times Sj$) and NG101 ($Se \times Sm$). The interspecific hybrid formation was further verified by individual species-specific PCRs (Muir et al., 2011; Reuben et al., 2013; Naseeb et al., 2021).

Aerobic shake-flask cultivations in synthetic medium with maltotriose as the sole carbon source

Cultivations of 50 ml SM + Mtt in shake flasks were performed in 500 ml Erlenmeyer flasks. Pre- and intermediate cultures and washing steps were conducted as the cultivations in the Growth Profiler upscaled for the intermediate cultures to 10 ml SM 2% (w/v) glucose in 100 ml Erlenmeyer flasks at orbital shaking of 250 rpm at 25°C. Growth was monitored by sampling every 2 h to measure OD$_{600}$ during the exponential growth phase of each strain. The maximum specific growth rate and lag phase duration were calculated for $S. pastorianus$ CBS 1513 and the interspecific hybrids NG92 ($Se \times Sj$) and NG101 ($Se \times Sm$). Samples for HPLC measurement were taken every 24 h to determine maltotriose consumption. Maltotriose and maltose were measured using an Agilent 1,260 LC equipped with a 1,260 Refractive Index Detector and an Bio-Rad Aminex HPX-42A Column (300 × 7.8 mm, 25 micron) operated at 75°C using MilliQ as eluent at a flow rate of 0.5 ml·min$^{-1}$.

Wort fermentations and aroma profiling

Pre-cultures of 10 ml YPD in 100 ml Erlenmeyer flasks were inoculated with single-cell colonies in triplicates and incubated at 20°C at 80–120 rpm for 24 h. Intermediate cultures of 30 ml 12 °P wort in 300 ml Erlenmeyer flasks were inoculated at 0.2 OD$_{600}$ and incubated at 20°C at 80–120 rpm for 48 h. The inoculum was calculated for each replicate by counting cells on a Neubauer Hemocytometer diluted with 50 mM EDTA to avoid flocculation and adjusted to 1.2 × 10$^8$ cells/mL. Simultaneously, the intermediate culture was gradually cooled to 12°C and the calculated inoculum volumes were mixed with fresh 12 °P wort to a final 200 ml volume with 0.1 mlL$^{-1}$ autoclaved Antifoam A reagent (Sigma-Aldrich, Germany) in 300 ml infusion bottles with a screw cap and septum (Avantor™, Netherlands) and a 3 cm stirring magnet. Release of CO$_2$ and pressure were controlled via a Microlance needle (Fisher Scientific, UK) covered with cotton to decrease contaminations. Fermentations were carried out at 12°C and the fermentation progress was monitored measuring weight loss through time. Samples were taken with a Microlance needle (Fisher Scientific, UK) and 5 ml were sampled for HPLC measurements of ethanol, glucose, maltose, and maltotriose.

Green beer was analyzed via gas chromatography /mass spectrometry (GC/MS) as described in Rollero et al. (2015) to determine concentrations of volatile compounds of interest. Aroma compound concentrations [mg·L$^{-1}$] were derived from triplicate biological fermentations with high reproducibility including values below and above the sensitivity of the GC/MS analysis per compound listed in Supplementary Table S10. Significant differences between each compound were calculated (p-values <0.05) with one-way ANOVA and Tukey’s multiple comparisons test (Supplementary Table S11). Z-scores were calculated per aroma compound by subtracting the mean of the sample from the observed value, divided by the standard deviation of the sample.

Results

HTS of Saccharomyces strains identified Saccharomyces mikatae strains exhibiting growth on maltotriose

The ability to utilize maltotriose is typically associated with ale $S. cerevisiae$ and $S. pastorianus$ strains, while limited utilization is seen throughout non-domesticated yeast species (Methner et al., 2019; Nikulin et al., 2020a). Nevertheless, relatively few yeast strains have been tested for this phenotype due to lack of appropriate screening tools and the high cost of the oligosaccharide in its pure form. Here, a previously described high-throughput assay for phenotyping microorganisms on solid media - PHENOS (Barton et al., 2018) was adopted to identify non-$cerevisiae$ Saccharomyces strains with the ability to grow on mineral medium with maltotriose as the sole carbon source. Nearly 200 Saccharomyces wild-type strains from various species, geographical locations, and origins were selected for the analysis and were initially tested on solid YP + Mtt at 30°C (Supplementary Figure S1) in two arrays – I and II (Supplementary Table S1). The strains tested comprise all eight currently known species of the Saccharomyces clade (except for $S. jurei$) and the domesticated Saccharomyces hybrids $S. pastorianus$ and Saccharomyces bayanus. Well-characterized control strains with known maltotriose phenotypes were included, i.e., $S. pastorianus$ CBS 1513 (Mtt$^+$) and $S. eubayanus$ CBS 12357$^+$ (Mtt$^-$). The Mtt$^+$ phenotype observed for the Mtt$^-$ control strain indicated that the medium required optimization (Supplementary Figure S1). Therefore, synthetic medium with maltotriose (SM + Mtt) and a different maltotriose batch with a higher purity were used in a next step to test the best performers in a single array including two $S. jurei$ strains (Supplementary Table S1: Array III). Performance was ranked based on maximum slope values generated via the PHENOS software (equivalent $\mu_{max}$). The value for the strain $S. eubayanus$.

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strain CBS 12357\textsuperscript{T} (Mtt\textsuperscript{−}) was set to 1 and the performances of all other strains were shown as fold-change in comparison (Supplementary Figure S2B). The top-performing strains included four non-\textit{cerevisiae} \textit{Saccharomyces} strains—the \textit{S. jurei} D5095\textsuperscript{T} and \textit{S. mikatae} LSYS65-1 strains which were recently reported as Mtt\textsuperscript{+} in parallel studies (Giannakou et al., 2021; Peris et al., 2022) and two \textit{S. mikatae} strains not described yet in literature for the phenotype tested—NBRC 11002 and NBRC 10997 (Supplementary Figure S2).

To eliminate any potential impurities in the components used in our solid medium (i.e., agar) that could have caused the residual growth of Mtt\textsuperscript{−} strains, all superior Mtt\textsuperscript{+} candidate strains and our Mtt\textsuperscript{−} and Mtt\textsuperscript{+} control strains were further analyzed in liquid SM + Mtt. To this end, the phenotyping was conducted in small volumes using the Growth Profiler. Under the respective conditions, only minimal growth was observed for CBS 12357\textsuperscript{T} (Mtt\textsuperscript{−}) contrasting the well-performing Mtt\textsuperscript{+} control, i.e., the strain \textit{S. pastorianus} CBS 1513 (Figure 1).

Real quantitative data such as maximum specific growth rates and lag phase durations could not be presented for the newly discovered non-\textit{cerevisiae} \textit{Saccharomyces} Mtt\textsuperscript{+} candidates due to flocculation of all four strains in SM. Nevertheless, all strains successfully grew on maltotriose as the sole carbon source. Three of the four superior Mtt\textsuperscript{+} strains exhibited a long lag phase while \textit{S. mikatae} NBRC 11002 interestingly switched metabolism from glucose (pre-culture) to maltotriose in SM without much delay similar to \textit{S. pastorianus} CBS 1513 (Figure 1).

Hybridizing \textit{Saccharomyces eubayanus} CBS 12357\textsuperscript{T} (Mtt\textsuperscript{−}) with \textit{Saccharomyces mikatae} NBRC 10997 (Mtt\textsuperscript{+}) or \textit{Saccharomyces jurei} D5095\textsuperscript{T} (Mtt\textsuperscript{+}) resulted in a drastically improved aerobic growth on maltotriose due to heterosis.

It was decided to combine the maltotriose utilization capabilities of the newly identified non-\textit{cerevisiae} \textit{Saccharomyces} Mtt\textsuperscript{+} candidates with the Mtt\textsuperscript{−} but cold-tolerant \textit{S. eubayanus} strain CBS 12357\textsuperscript{T} in a hybrid and check for the heterosis effect in a comparative growth experiment in liquid medium. To this end, it was intended to introduce auxotrophic markers in the parental strains; however, complementary auxotrophs to facilitate mating by complementation were not successfully isolated. Instead, it was decided to rapidly generate stable haploids via genetically engineering the HO endonuclease to abolish self-mating (Strathern et al., 1982). This approach allows the generation of MAT\textsuperscript{a}/MAT\textsuperscript{α} ho/ho allodiploids which could be further exploited for future quantitative genetic studies on multiple generations of random mating of allotetraploid hybrids with restored fertility (Naseeb et al., 2021). Subsequently, de novo hybrids between \textit{S. eubayanus} CBS 12357\textsuperscript{T} and \textit{S. jurei} D5095\textsuperscript{T} and between \textit{S. eubayanus} CBS 12357\textsuperscript{T} and \textit{S. mikatae} NBRC 10997, i.e., NG92 (Se × Sj) and NG101 (Se × Sm) were successfully constructed.

To record the kinetics of sugar consumption and ethanol formation in addition to biomass formation of the strains during aerobic growth in liquid SM + Mtt, a higher culture volume was
required for frequent sampling of culture supernatants. Thus, the
previous Growth Profiler experimental setup (Figure 1) was
upscaled to 50 ml cultures in shake flasks which were inoculated
with the de novo hybrids - NG92 (Se × Sj) and NG101 (Se × Sm)
in parallel with the corresponding parental strains and the
S. pastorianus strain CBS 1513 as Mtt1 reference. As expected, the
S. pastorianus strain CBS 1513 completed maltotriose
consumption after 48h of cultivation in SM + Mtt (Figure 2A). In
contrast, the hybrids NG92 (Se × Sj) and NG101 (Se × Sm) only
consumed 25 and 35%, of sugar, respectively, after 120h of
cultivation (Figures 2B, C). Interestingly, the two hybrids did not
flocculate in comparison to their respective S. mikatae/S. jurei
parental strain allowing growth rate determination during
exponential phase. Remarkably, the two de novo hybrids
outperformed both parental strains exhibiting growth rates [h−1]
comparable to the S. pastorianus strain CBS 1513 and a shorter lag
phase [h] compared to the parental Mtt1 strains S. jurei D50955
and S. mikatae NBRC 10997 (Figures 2A–C). NG92 (Se × Sj)
displayed a significantly shorter lag phase than NG101 (Se × Sm;
Figures 2B, C).

Notably, the S. pastorianus strain CBS 1513 produced ethanol
from maltotriose under the applied conditions. After 24h of
cultivation, an ethanol concentration of 4g L−1 was detected
(Figure 2D). The fact that there was no remaining ethanol after
48h demonstrated that the ethanol formed was afterward
consumed. However, no ethanol formation could be detected in
either of the two de novo hybrids NG92 (Se × Sj) and NG101
(Se × Sm; Figures 2E, F). Probably, these strains indeed did not
show any overflow metabolism under the tested conditions or the
appropriate time point representing the ethanol production peak
was simply missed during sampling.

The de novo hybrids NG92 (Se × Sj) and
NG101 (Se × Sm) completely fermented maltose, in contrast to their
corresponding Saccharomyces
jurei/Saccharomyces mikatae parents,
but not maltotriose in lager brewing conditions

Eventually, it was interesting to test the novel hybrids under
conditions which are relevant in lager brewing. To this end, brewer’s
wort fermentations at 12°C were conducted in lab scale with the
constructed de novo hybrids—NG92 (Se × Sj) and NG101 (Se × Sm),
all parental strains—S. eubayanus CBS 12357T, S. jurei D50955,
and S. mikatae NBRC 10997 as well as S. pastorianus CBS 1513. The latter
strain was added as a reference since it is regularly used in industrial
lager beer production (Figure 3). Within the first 48h, all strains
rapidly fermented glucose (Figure 3B) including the residual
amounts of fructose and sucrose estimated by the calculated total
sugar consumption via weight/CO2 loss measurements
( Supplementary Table S8). After this time point, S. pastorianus CBS
1513 and S. eubayanus CBS 12357T, NG92 (Se × Sj), and NG101
(Se × Sm) completely utilized maltose without an apparent lag phase,
while the parental S. jurei D50955 and S. mikatae NBRC 10997
strains did not utilize the disaccharide at all. Notably, the de novo
hybrids exhibited a slower rate of maltose fermentation(140,153),(866,893)
S. pastorianus CBS 1513 and S. eubayanus CBS 12357\textsuperscript{T} and required additional 24–48 h to complete the utilization of the sugar (Figure 3C). As expected, the domesticated lager hybrid (S. pastorianus) consumed maltose most rapidly and ethanol formation of 4.4% alcohol by volume (ABV) was achieved. Moreover, S. pastorianus CBS 1513 co-consumed maltose and maltotriose once glucose was depleted as previously described (Magalhães et al., 2016; Figures 3C, D). The two hybrids, NG92 (Se × Sj) and NG101 (Se × Sm), could not utilize maltotriose during the conducted wort fermentations (Figure 3D) similar to S. eubayanus CBS 12357\textsuperscript{T} only reaching an ABV of ~3.5% (Figure 3A) although they efficiently grew on maltotriose as the sole carbon source in the shake-flask cultivations at 25°C, with comparable maximum specific growth rates to S. pastorianus CBS 1513 (Figures 2A–C).

Green beer produced by de novo hybrids NG92 (Se × Sj) and NG101 (Se × Sm) showed increased concentrations of fruity flavor esters

The green lager beers produced by the de novo hybrids NG92 (Se × Sj) and NG101 (Se × Sm) as well as their respective S. jureii/S. mikatae parent and the common parent S. eubayanus CBS 12357\textsuperscript{T} (Figure 3) were comparatively analyzed with GC/MS for their aroma profiles. In addition, the lager strain S. pastorianus CBS 1513 was used as a reference. Green beers obtained with the de novo hybrids showed different concentrations of aroma compounds compared to their parental strains (Supplementary Table S9). In fact, heterotic production of desired esters was achieved, especially for NG101 (Se × Sm; Supplementary Table S9). We compare here the actual aroma concentration values detected keeping in mind that the parental strains – S. jurei D5095\textsuperscript{T} and S. mikatae NBRC 10997 could not ferment maltose and maltotriose, therefore, they produced concentrations close or below the lower threshold of measurement. Accurate comparison between the S. eubayanus strain CBS 12357\textsuperscript{T} and the interspecific hybrids is possible reaching an equal final ABV of 3.4–3.5% (Figure 4). Notably, enhanced production of ethyl esters such as ethyl hexanoate (green apple) was observed for NG92 (Se × Sj) which was 2.5-fold higher in comparison to the S. eubayanus CBS 12357\textsuperscript{T} parent, while NG101 (Se × Sm) produced this aroma compound in amounts above its flavor threshold in lager beer (Figure 4). Moreover, ethyl octanoate (apricot) concentration levels were ~2-fold higher for both hybrids compared to CBS 12357\textsuperscript{T} (Figure 4). Comparable concentrations...
were detected for different acetate esters tested between NG92 (Se × S), NG101 (Se × Sm), and CBS 12357 †. Interestingly, higher concentrations of isoamyl acetate (banana; 5.22 mg L$^{-1}$) above the reported flavor threshold in beer and 2-phenylethyl acetate (3.75 mg L$^{-1}$) close to its threshold of perception were determined for the NG101 (Se × Sm) hybrid compared to the industrial lager hybrid – S. pastorianus CBS 1513 (Supplementary Table S9). Similar amounts were identified between all hybrids and S. eubayanus CBS 12357 † for favorable higher alcohols such as 2-phenylethanol (rose, floral) and propanol (spicy, hard). However, the off-flavor 2,3-dimethylbutan-1-ol (roasted onion) associated typically with wine fermentations was produced with a slightly higher concentration in the de novo hybrids (Supplementary Table S9). Undesired medium-chain fatty acids (MCFA) were detected for all strains tested with significantly higher concentrations of caprylic acid (goaty) produced by S. pastorianus CBS 1513 albeit below its taste threshold (Supplementary Table S9). Although we present here a diverse set of aroma compounds with interesting heterotic production of desired fruity esters, the combined aroma profile could not be assessed in a sensory analysis due to the use of genetic engineering to construct the strains.

**Discussion**

The use of wild yeasts and de novo hybrids in brewing is an attractive approach toward diversifying the lager beer style. However, the challenge is to meet pleasant taste with fermentation performance at the relative cold fermentation temperatures during lager beer brewing. The consumption of the wort sugar maltotriose is an important trait; however, non-conventional Saccharomyces strains able to utilize maltotriose have not been reported at the time when the current study was initiated.

Our HTS identified so far uncharacterized S. mikatae Mtt* wild strains as well as the recently reported Mtt* S. mikatae LYS65-1 strain (Peris et al., 2022). Parallel studies conducted by other authors demonstrated that the two S. jurei strains TUM 629 and D5095 † are also able to utilize wort maltotriose to a certain extent (Giannakou et al., 2021; Hutzler et al., 2021) and that some S. mikatae strains are able to grow on this sugar (Peris et al., 2022). The current study included the strain S. jurei D5095 † and confirmed its Mtt* phenotype. The S. jurei D5095 † (Mtt*) strain and one of the identified Mtt* S. mikatae strains in this study (NBRC 10997) were used to construct novel hybrids with the S. eubayanus strain CBS 12357 †. We demonstrated several clear heterosis effects in the hybrids as discussed below.

Our HTS of ~200 strains of the clade Saccharomyces for growth on maltotriose as the sole source of carbon revealed limitations when conducted on solid medium. In fact, the negative control strain S. eubayanus CBS 12357 † showed significant growth in the PHENOS phenotyping. Still, the latter method successfully identified superior Mtt* isolates of non-domesticated yeasts. Their Mtt* phenotypes were afterward confirmed in a liquid medium via the Growth Profiler.

Our finding that S. mikatae strains exhibit growth on maltotriose as the sole carbon source provides an example of the high intraspecies phenotypic diversity uncovered recently for the species despite its low genetic diversity (Peris et al., 2022). However, only a limited number of strains have been tested so far for wort fermentability. In fact, Nikulin et al. (2018) showed that neither α-glucoside (maltose and maltotriose) in brewer’s wort was utilized by the S. mikatae type strain (IFO 1815). Moreover, authors only tested the strain’s performance at 12°C which, in the current study, turned out to be unfavorable for the utilization of the respective wort sugars by S. mikatae.

So far, S. mikatae strains have only been isolated in East Asia even though evidence of the species’ presence in Europe was provided by sequencing the ITS1 region from DNA obtained from the mycobiome of soils surrounding trees in the Italian alps (Alsammar et al., 2019). Interestingly, S. jurei is the most closely related species to S. mikatae (Naseeb et al., 2018) and strains of S. jurei have already been isolated in Europe. The existence of Mtt* isolates in both species seems to be in contrast to all other non-domesticated Saccharomyces species. Although more data are required to verify this, one might hypothesize that the ability to grow on maltotriose could have been obtained before the evolutionary split of the two species.

Phylogenetic distribution of MAL genes throughout the Saccharomyces clade suggests that maltotriose transport is the limiting factor (Day et al., 2002; Baker and Hittinger, 2019), mediated by Agt1 (Alves et al., 2008) and Mtt1 (Dietvorst et al., 2005), while certain diastatic yeasts strains can degrade the sugar extracellularly prior to transport (Alves et al., 2018). Hutzler et al. (2021) confirmed maltotriose transmembrane transport in the S. jurei strain TUM 629 employing radiolabeled substrate. The authors conducted a genetic analysis on the respective strain and identified a gene with 82.6% sequence identity to S. cerevisiae AGT1, which can potentially encode for a maltotriose permease. This might also apply to S. jurei D5095 †, considering the low sequence divergence of the two strains (0.1%). The three new Mtt* S. mikatae strains identified here (LYS65-1, NBRC 11002, and NBRC 10997) are interesting candidates to further study the genetic basis underlying the Mtt* phenotypes in the species S. jurei and S. mikatae. For whole-genome sequencing, it is recommended to use third-generation sequencing to properly assemble sub-telomeric regions which cannot be dissected by Illumina sequencing. As known from other Mtt* strains of the Saccharomyces clade, the sub-telomeric regions usually contain the MAL genes.

One might further question whether the genetic resources causing the Mtt* trait of the ale yeast S. cerevisiae can be replaced by the newly discovered Mtt* wild strains (S. jurei and S. mikatae) when hybridized with the cold-tolerant S. eubayanus CBS 12357 †. Enhancing traits relevant in brewing via interspecific Saccharomyces hybrids has been successfully applied in several previous studies (Hebly et al., 2015; Krogerus et al., 2015).
We hypothesized that heterosis regarding the Mtt\(^+\) phenotype could be achieved in such hybrids via synergistic actions of structural and regulatory proteins involved in maltotriose metabolism. Regarding the two sub-genomes of \(S.\) pastorianus (\(S.\) cerevisiae and \(S.\) eubayanus), a regulatory crosstalk between MALR and MALT genes has previously been demonstrated in a de novo \(S.\) pastorianus hybrid and provided a possible explanation for the efficient maltotriose fermentation of the domesticated lager yeast (Brouwers et al., 2019a). The common parent, \(S.\) eubayanus CBS 12357\(^T\), does not contain functional maltotriose transporters but has been shown to exhibit evolvability to the trisaccharide indicating the existence of genetic resources (Baker and Hittinger, 2019; Brouwers et al., 2019b). Interestingly, the de novo hybrids between Mtt\(^-\) \(S.\) eubayanus CBS 12357\(^T\) and Mtt\(^+\) \(S.\) jurei D5095\(^T\)/\(S.\) mikatae NBCR 10997 successfully displayed heterosis with regard to their aerobic growth performance on maltotriose as the sole carbon source. Both hybrids, NG92 (Se \(\times\) Sj) and NG101 (Se \(\times\) Sm) outperformed the respective parents in terms of this trait, and both exhibited maximum specific growth rates comparable to the industrial \(S.\) pastorianus CBS 1513 strain.

We presume that, in the de novo hybrids, an increased expression of MALR regulatory genes originating from the \(S.\) eubayanus CBS 12357\(^T\) genome could positively regulate potential transporter genes in the sub-genomes of \(S.\) mikatae NBRC 10997 and \(S.\) jurei D5095\(^T\). Notably, there was a significant difference in the lag phase duration between NG92 (Se \(\times\) Sj) and NG101 (Se \(\times\) Sm) upon this switch to maltotriose which might have been caused by differences in aroma production by the generated de novo hybrids NG92 (\(S.\) eubayanus CBS 12357\(^T\) \(\times\) \(S.\) jurei D5095\(^T\)/Se \(\times\) Sj) and NG101 (\(S.\) eubayanus CBS 12357\(^T\) \(\times\) \(S.\) mikatae NBRC 10997/Se \(\times\) Sm) and their common parental strain \(S.\) eubayanus CBS 12357\(^T\). Colors show the range of the calculated Z-scores per aroma compound indicating production values above average, average, and below average shown with blue, yellow, and red, respectively. The aroma compounds are grouped by acetate esters, ethyl esters, alcohols, and acids (medium-chain fatty acids/ MCFAs). Flavor thresholds in beer values (shown in brackets) were obtained from Meilgaard (1982) (a); Meilgaard (1975a) (b); Meilgaard (1975b) (c); Harrison (1970) (d). Corresponding aromas/flavors for each volatile compound tested were obtained from The Good Scent Company Information System (2022) (e); Swiegers et al. (2005) (f); PubChem (2022) (g); Dunley et al. (2009) (h); Blanco et al. (2016) (i). The calculations are based on aroma compound concentrations (mg\(\text{L}^{-1}\)) from triplicate biological fermentations with high reproducibility including values below and above the sensitivity of the GC/MS analysis. Detailed quantitative data of the aroma compounds with standard deviation of all strains including the aroma profile of the reference \(S.\) pastorianus CBS 1513 strain are presented in Supplementary Table S9. Z-scores generated from concentrations below or just above the threshold of measurement are marked with an asterisk (*)

**Figure 4**
Comparative analysis of aroma production by the generated de novo hybrids NG92 (\(S.\) eubayanus CBS 12357\(^T\) \(\times\) \(S.\) jurei D5095\(^T\)/Se \(\times\) Sj) and NG101 (\(S.\) eubayanus CBS 12357\(^T\) \(\times\) \(S.\) mikatae NBRC 10997/Se \(\times\) Sm) and their common parental strain \(S.\) eubayanus CBS 12357\(^T\). Colors show the range of the calculated Z-scores per aroma compound indicating production values above average, average, and below average shown with blue, yellow, and red, respectively. The aroma compounds are grouped by acetate esters, ethyl esters, alcohols, and acids (medium-chain fatty acids/ MCFAs). Flavor thresholds in beer values (shown in brackets) were obtained from Meilgaard (1982) (a); Meilgaard (1975a) (b); Meilgaard (1975b) (c); Harrison (1970) (d). Corresponding aromas/flavors for each volatile compound tested were obtained from The Good Scent Company Information System (2022) (e); Swiegers et al. (2005) (f); Dunley et al. (2009) (h); Blanco et al. (2016) (i). The calculations are based on aroma compound concentrations (mg\(\text{L}^{-1}\)) from triplicate biological fermentations with high reproducibility including values below and above the sensitivity of the GC/MS analysis. Detailed quantitative data of the aroma compounds with standard deviation of all strains including the aroma profile of the reference \(S.\) pastorianus CBS 1513 strain are presented in Supplementary Table S9. Z-scores generated from concentrations below or just above the threshold of measurement are marked with an asterisk (*).
in the sub-genome interactions between the common parent *S. eubayanus* CBS 12357\(^1\) and the *S. jurei* and *S. mikatae* parent, respectively.

Regardless of the remarkable maximum growth rates in SM + Mtt, it remains unknown why biomass formation of the *de novo* hybrids ended before maltotriose was depleted which was in clear contrast to *S. pastorianus* CBS 1513, which completely consumed the trisaccharide exhibiting a comparable growth rate. The latter partially assimilated maltotriose in a fermentative manner (as visible by ethanol production) in the aerobic shake-flask experiments. Simple shake-flask cultivations are known for limited oxygen supply. Moreover, no control over important variables such as pH is possible (Link and Weuster-Botz, 2011). Oxygen limitations might have resulted in a limited supply of ATP required to transport maltotriose through the cell membrane via the presumed maltotriose transporter in the two hybrids NG92 (*Se × Sj*) and NG101 (*Se × Sm*). Notably, the Agt1 permease has been shown to be an active maltotriose H\(^+\) symporter (Stambuk et al., 1999). A low substrate affinity of maltotriose transporters might contribute to the sudden growth termination of the *de novo* hybrids.

While aerobic growth on maltotriose as the sole carbon source is an interesting trait in the context of phylogeny, successful fermentation of the sugar to alcohol is of much greater importance for brewers. While strains of the domesticated species *S. pastorianus* strains are well known for the latter trait, the *de novo* hybrids NG92 (*Se × Sj*) and NG101 (*Se × Sm*) did not ferment maltotriose in spite of their ability to aerobically assimilate maltotriose. Nevertheless, the hybrids completely fermented maltose which contrasted with their corresponding *S. jurei* *S. mikatae* parents even though the rates were lower compared to that of the other co-parent, *S. eubayanus* CBS 12357\(^2\). An improvement of the wort sugar fermentation rates could be achieved via prior adaptation of the strains to wort. In fact, re-pitching in maltose-rich medium combined with cell viability monitoring were vital requirements to adapt *S. paradoxus* for wort fermentations (Nikulin et al., 2020b). Moreover, pre-adaptation to maltotriose was shown to be essential to achieve partial fermentation of the trisaccharide in wort by *S. jurei* TUM 629 (Hutzler et al., 2021). These findings suggest that further adaptive evolutionary experiments with NG92 (*Se × Sj*) and NG101 (*Se × Sm*) could be a promising approach to fully exploit their potential to ferment brewher’s wort. Nevertheless, the genome stability of the genetically engineered hybrids must be evaluated and directed evolution might be necessary to reach an industrial potential. The use of genetic modifications gave a convenient and rapid opportunity to study the fermentative and aromatic potential of such novel *de novo* hybrids in the lab environment. Still, alternative mating methods without genetic manipulations are strongly preferred if hybrids will be used in large-scale industrial applications in beer (Nakatomi and Gunge, 1972; Alexander et al., 2016; Gorter De Vries et al., 2019).

The most valuable traits of using wild strains and novel hybrids for beer consumers are the exotic aromas and flavors present in the beer produced with such yeast strains. In the hybrids constructed here with *S. eubayanus*, the parental wild *S. jurei* and *S. mikatae* strains contributed to the aroma profile. There was a heterotic production of desired fruity aroma compounds. Both NG92 (*Se × Sj*) and NG101 (*Se × Sm*) revealed favorable heterotic production of ethyl octanoate (apricot) and ethyl hexanoate (green apple). The latter was already outlined in the study of Hutzler et al. (2021) for contributing to the tropical final profile caused by *S. jurei* TUM 629 as detected via a sensory panel. Other desirable aromatic compounds in brewing such as 3-methylbutyl acetate (banana) and 2-phenylethyl acetate (rose, fruity) exhibited higher concentration in both hybrids compared to the commercial *S. pastorianus* CBS 1513 (Supplementary Table S9). The concentration of 2-phenylethanol was also increased in both *de novo* hybrids and increased concentrations of this compound have been highlighted to mask unwanted aroma compounds such as 4-VG notes typical for wild strains (Bamforth, 2020). The determination of important off-flavors such as diacetyl (butter-like; Duong et al., 2011) or 4-VG (clove-like; Coghe et al., 2004) was not in the scope of this study. Although the genetically modified organism (GMO)-nature of the constructed hybrids hampered any sensory evaluations, the measured concentrations of aroma compounds suppose a potential exotic tropical final aroma profile.

**Conclusion**

The increase in the consumer demand for aromatic diversity in beer can be met in lager beer through interspecific hybridization of wild *Saccharomyces* yeast species. In such attempts, the utilization of maltotriose by the generated hybrids is a relevant trait for brewers. Here, we report maltotriose-utilizing strains from the species *S. mikatae* and confirmed the Mtt\(^+\) phenotype recently identified by others in strains of *S. jurei* (D5095\(^3\)) and *S. mikatae* (LSYS65-1). We constructed two *de novo* hybrids: (i) between an Mtt\(^+\) *S. mikatae* strain isolated in the current study (NBRC 10997) and the Mtt\(^−\) but cold-tolerant *S. eubayanus* CBS 12357\(^2\) and (ii) between the Mtt\(^−\) *S. jurei* strain D5095\(^3\) and the *S. eubayanus* strain CBS 12357\(^2\). Both hybrids displayed heterosis on maltotriose and outperformed both parents regarding aerobic growth on maltotriose synthetic medium achieving growth rates comparable to the industrial reference strain *S. pastorianus* CBS 1513. In contrast to their *S. mikatae*/*S. jurei* parents, the *de novo* hybrids completely fermented maltose in wort fermentations in a lager brewing setting and produced enhanced amounts of favored fruity esters. Although the hybrids failed regarding the fermentation of maltotriose, there is the potential to improve this relevant trait as well as the rate of maltose fermentation by future adaptive laboratory evolution. The current study might therefore pave the way toward non-*cerevisiae* *Saccharomyces* hybrids able to completely ferment all wort sugars and brew exotic lager beers. By using complementary auxotrophic mutants and/or different physiological traits of the partners, non-GMO hybrids could be produced which could contribute to the diversification of lager beers in the future.
Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions

NG conducted the experimental work and drafted the manuscript. AC-M, SW, and SA provided experimental assistance. EL and EN provided support in experimental setup and writing guidance. NK provided support in experimental setup and critical revision of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

NK was employed by HEINEKEN Supply Chain B.V.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1025132/full#supplementary-material
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